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(71) Applicant *(for all designated States except US)*: ENTERON, L.P. [US/US]; P.O. Box 4988, Oak Brook, IL 60522 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: CALENOFF, Emanuel [US/US]; 750 North Rush Street, No. 3402, Chicago, IL 60611 (US).

(74) Agent: MARTIN, Alice, O.; Brinks Hofer Gilson & Lione, NBC Tower – Suite 3600, 455 North Cityfront Plaza Drive, Chicago, IL 60611–5599 (US).

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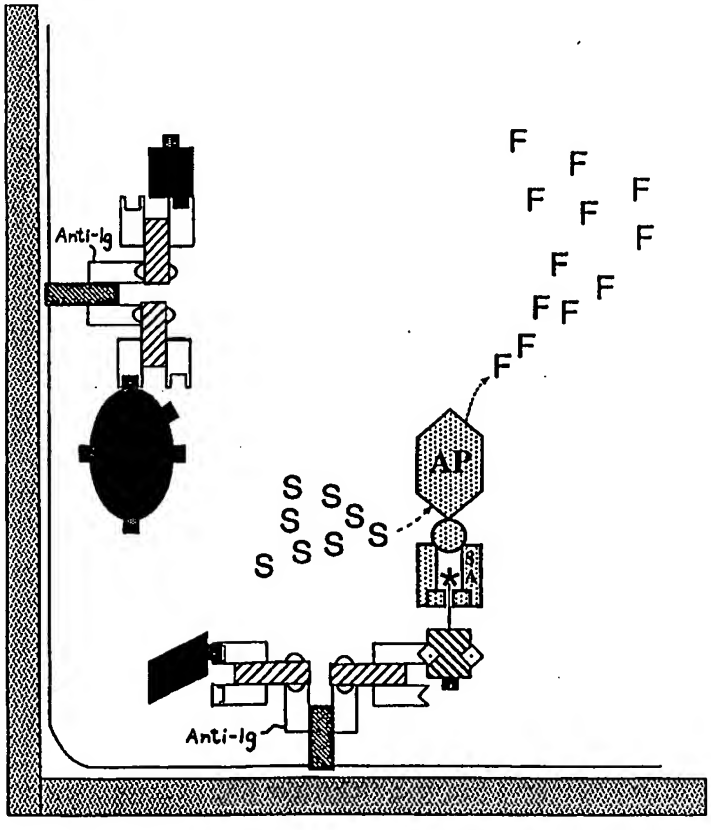
(57) Abstract

A new capture assay method employs novel compositions of reformulated antigens including epitopes specific for an organism that is a target of the assay, and epitopes specific for an allergen, wherein each antigen is present in equivalent amounts, and to which non–specific epitopes are added to remove non–specific binding as a confounding factor in the assay. The assay is suitable for detection of immunoglobulins directed to specific organisms, such as micro–organisms and parasites, and for allergens. For example, specific IgG in combination with IgE levels are used to detect *Helicobacter pylori* and *Chlamydia pneumoniae* and to monitor response to therapy.



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ORGANISM-SPECIFIC AND ALLERGEN-SPECIFIC ANTIBODY CAPTURE ASSAY AND COMPOSITIONS FOR DETECTION OF DISEASE-CAUSING ORGANISMS AND ALLERGENS

This invention relates to a new capture assay for detecting immunoglobulins (antibodies) directed to specific epitopes, in particular epitopes derived from organisms such as microorganisms and small parasites, and epitopes specific for an allergen, to novel antigen compositions for use in such assays, and to methods of preparing the compositions.

Assays to detect IgA, IgD, IgE, IgG and IgM antibodies directed to specific epitopes have used either an entire organism, an entire antigen molecule, mixtures of antigenic molecules, or antigenic portions of individual molecules as part of the assays. Various degrees of purification of antigens have been employed. Methods to detect antigen-antibody complexing from which the presence of specific epitopes are inferred, are well known and include sandwich-type immunoassays, capture-type immunoassays, competition-type immunoassays, homogeneous-type immunoassays and others. (Tijssen, 1985) However, significant problems with interspecies cross-reactivity and binding among antibodies, prevent the specificity and sensitivity of immunoassays for disease causing organisms from reaching acceptable levels.

Antibody capture assays are sometimes referred to as "reverse" ELISAs. Antibody capture assays have some advantages over indirect enzyme-linked immunosolvent assay (ELISA) methods, but the problem of cross-reactivity and non-specific reactions has not been solved satisfactorily. Attempts were made in some studies to block common epitopes by incubating an antigen on a solid phase

with antiserum to related organisms, or by adding heterologous, heat denatured organisms to consume antibodies reacting with common epitopes.

Immunological methods (assays) have been reported to detect the presence of specific disease-causing or allergy causing organisms in biological fluids. Diagnostic kits comprising antigen, antibody, label, and anti-antibodies have been described based on some of the assays. Superficially, some of the assays seem similar to each other and to methods of the present invention. However, more sophisticated scrutiny reveals major differences in components of the assays, specifically, the compositions of antigens used, mechanisms for blocking binding sites on antigens or antibodies to increase sensitivity and specificity of the assays, configuration of the elements of the assay relative to a support, and which element is labeled, wherein "element" includes antigen, antibody, and an agent to capture the antibody. An additional difference among immunological detection methods is the type of antibody used or detected, e.g. type of immunoglobulin. Finally, previous immunoassays designed to detect organisms causing disease or allergy, to detect the presence of the associated disease, and to monitor the effects of treatment, have not been clinically successful and have not been applicable in general to a variety of organisms.

H. pylori, an organism associated with gastrointestinal disease including gastric cancer, is one specific target of such assays. With regard to antigenic compositions, "antigens" selected from *H. pylori* (initially termed *C. pylori*) have included the entire organism, only the surface antigens, the outer membrane proteins, and various fractions of antigenic proteins, wherein certain fractions are

selected and/or pooled for use in an assay. Kits comprising antigen, antibody, label, and anti-antibodies have been described. None of the reported assays are acceptable for clinical use.

Alemohammad (1993) reviewed problems in developing non-invasive methods to detect *H. pylori*. Major problems with ELISA were identified as "the source, type and characteristics of the antigen used." Col. 3, lines 7-9. For example, Evans et al. (1992) relate an antigen which is derived from *C. pylori* and is said to be "purified," "high molecular weight," (300-700 kD) and "cell-associated." In the Evans method, the antigens are immobilized on a solid support, the antibodies in a biological sample to be tested complex with the immobilized antigens, the antibody detected is IgG, and labeled anti-IgG is added to detect the antigen-antibody complexes. Blocking of the non-specific binding to the solid support is accomplished by providing excess of BSA (bovine serum albumin). The "antigen" used in the assay is prepared by pooling fractions selected on the basis of high molecular weight, and in a preferred embodiment, urease activity. Evans erroneously suggested that mere presence of *H. pylori* reactive IgG is indicative of disease. However, elevated levels of IgG, IgA or IgM directed to *H. pylori* in a biological fluid sample from a subject indicates only that a subject is carrying the bacteria, not necessarily that the individual is the affected with a disease related to the infection.

Hirschl (1990) attempted to detect *H. pylori* using crude, impure antigen preparations (ultracentrifuged cell sonicates or acid glycine extracts) with a 120 kD protein for serodiagnosis. Only IgG was used in the assay. He acknowledged

a theoretical advantage for using whole cell sonicates to expose a maximum number of surface antigens, but stated that such compositions "increased the risk of non-specific binding of immunoglobulins and of cross reactions with related species." (page 512)

Newell (1990) prepared antigens from *C. pylori* by separating proteins by a chromatographic technique, removing fractions responsible for non-specific cross-reactions and optionally combining other fractions. Blaser (1989) proposed a variety of fractional components of *C. pylori* as antigenic compositions, including components from many strains. Cover (1995) advocated use of the tag A protein or fragments thereof in an immunoassay for *H. pylori*.

Reid used an immunoassay configuration in which antigen is immobilized on a support, then antibodies are added from a serum to be tested, than a labeled anti-IgE is added. Reid believed an improvement was the use of a mouse monoclonal antibody to human IgE. The problem of IgG interfering with the IgE assay was addressed by "dilution, serial transfer, and cumulative counting." (Abstract)

Even methods that focused on use of a library of isolated and purified antigens specific for an organism, although an improvement on previously reported methods, were not completely satisfactory. Calenoff (1996) did not employ all, or even many, specific epitopes present in the organism because not all the epitopes were included in the antigenic molecules selected. Also, some methods of antigen preparation were not easily reproducible, e.g. if strains of microorganism mutated, causing the appearance or loss of specific epitopes, new

libraries may be needed and these were not easy to prepare.

With regard to immunoassays to detect agents causing other diseases or deleterious conditions, Calenoff (1989) taught a method of assaying for allergen specific IgE wherein the presence of specific IgE indicated the presence of an allergy. In the assay, an allergen is bound to a solid support, then contacted with serum from a subject. If allergen (e.g. mold) specific IgE is present in the serum, the binding of IgE to the mold antigen is detected by labeled anti-E antibody.

Despite some progress in development of immunoassays for specific diseases or conditions, or specific allergens, significant levels of misclassification still plague the reported assays and make them suspect for clinical use to replace more invasive and expensive diagnostic procedures (such as esophagogastroduodenoscopy and biopsy for *H. pylori* detection). Therefore, better methods are needed to increase the sensitivity and specificity of antigen-antibody complexing assays to detect the presence of organism-specific or allergen specific antibodies in a biological fluid, in particular microorganism-specific and small parasite-specific antibodies. Improved assays for detecting the presence of infecting or colonizing organisms and their deleterious effects require different antigenic compositions, blocking or quenching mechanisms, and/or assay configurations such as organization of elements in the assay relative to a support. The assays also need to be accurate, reproducible, and relatively easy to prepare and use.

SUMMARY OF THE INVENTION

The present invention attacks existing problems in clinical immunoassays by presenting new antigen compositions and a novel and effective solution to the problem of cross-reactivity. A new capture antibody assay for immunoglobulins is directed to epitopes that are specific to a target organism or allergen and are immunoreactive. Novel antigen compositions for use in such assays; methods of preparing the compositions; and methods for blocking reactions that would decrease accuracy of the assay, are also aspects of the invention.

The present invention relates compositions neither taught nor suggested in the art-compositions that include substantially all the antigenic sites (epitopes) present in an organism, or allergen, reformulated with a quantitative factor so that the epitopes are present in the compositions in approximately equivalent amounts, so that specific epitopes that are present in relatively small quantities in the organism or allergen have as good a chance of being discriminated as those present in greater proportions. Labeling of antigens is indiscriminate, that is, all antigenic molecules prepared from the target organism or allergen are labeled. The improved performance of these compositions in immunoassays reflects a greater number of specific epitopes available compared to other reported compositions used for similar purposes. Compositions of the present invention are also easier to prepare than other reported antigenic compositions because extensive purification is not required, and the specificity of the epitopes does not need to be predetermined.

The specific epitopes included in a mixture of antigens derived from the target organism or allergen are expected to bind specifically to the antibodies in a

biological sample, if indeed, the biological sample being tested has antibodies which react with the specific epitopes. If the sample comes from a subject infected or colonized with the target organism, and the subject has produced an immunological response to the organism, immunoglobulins that recognize epitopes on the organism should be present. For some diseases or conditions, the mere presence of a target organism may not be sufficient to establish presence of an associated disease or condition. In the case of gastric ulcers, for example, if *H. pylori* has not produced an IgE-mediated immune response, the organism may be present and detectable by specific IgG, but associated disease is absent as evidenced by lack of IgE specific for *H. pylori*.

In addition to novel antigen compositions, another novel aspect of the invention is the means by which non-specific epitopes present in a target organism or allergen are prevented from diminishing the accuracy of the assay to detect antibodies specific for the target organism or allergen. Nonlabeled reformulated, or non-reformulated antigen is used to quench (block) the non-specific, antibody-based recognition of a non-specific epitope or plurality of epitopes on a labeled antigen of interest. To be effective, the non-labeled antigen shares non-specific epitopes with the labeled antigens, i.e. has homologous epitopes. The presence of non-specific epitopes on non-labeled antigen ties up non-specific binding sites and reduces the chance of non-specific epitope binding of immunoglobulin to the non-specific epitopes on the labeled antigens. Recognition of non-specific epitopes is thereby removed as a confounding factor in an assay for a specific organism. Homologous non-specific epitopes are those that are structurally similar enough to

successfully compete with non-specific epitopes on molecules from the target organism for binding with antibodies in a biological sample. Whether to reformulate the non-specific epitope mixture or use it without reformulation is based on convenience and cost. It generally takes more material to achieve the same quenching effect if the mixture is not reformulated. Also, adding too much non-reformulated antigen may confound clear, reliable performance of the assays of the present invention because some molecules may be "sticky" and when in excess may act like glue attracting labeled molecules to the solid support.

The detection method of this invention comprises attaching an anti-immunoglobulin to a support, generally an insoluble support, and exposing a biological fluid sample to the support so that some or all antibodies of the fluid can be captured by the anti-immunoglobulin of the support. Preferably the quantity of anti-Ig applied is known. Monoclonal antibodies or polyclonal antibodies that are affinity purified are suitable as anti-immunoglobulins, as are other suitable materials such as protein A or G. Cross recognition of other Ig types is minimized because only the Ig type corresponding to the binding agent on the support is detected e.g. if anti-IgE is on the support, IgG will wash out before the assay is evaluated. A preferred capture assay depends on a covalent linkage between the capture antibody and the surface so washing the system does not disrupt the binding.

After removing the uncaptured antibodies by washing away the biological fluid sample, a mixture containing the labeled reformulated antigen and non-labeled reformulated or non-reformulated antigen, the latter usually extracted from

an antigen source or sources which is(are) closely related taxonomically to the source of the labeled antigen, is added to the assay. The captured antibodies bind the labeled antigen and unlabeled antigen of the antigen mixture. After allowing non-bound antigen solution to wash away, only the labeled antigen is detected, thereby identifying the presence of antibodies from the biological fluid sample which are specific for epitopes characteristic of a particular target organism.

Another aspect of the invention is that a combination of values from different immunoglobulin types is a preferred type of diagnostic algorithm, e.g. combining IgG and IgE values. A preferred type of combination is to multiply target specific levels of IgG by target specific levels of IgE.

To elaborate, aspects of the present invention include:

1. antigen extraction;

2. reformulating of the extracted antigen; and

3. a novel capture assay to detect antibodies specific for an individual target organism, or allergen, an assay that employs non-specific epitopes from organisms or allergens related to the target organism to address problems of non-specificity that plague other immunoassays.

An "antigen" is a molecule containing one or more epitopes. As used herein, antigen refers to proteins or protein-containing molecules. Antigens may have a plurality of epitopes, that is, sites that individually complex with different antibodies. Some epitopes are specific for an individual microorganism or other antigen source; others are non-specific, that is, may appear on molecules provided by other antigen sources. The invention uses all available specific epitopes on an

antigen of interest, and blocks from the specific reaction, non-specific epitopes which are detrimental to high sensitivity and specificity of the assay. However, most of the antigens from a target organism have no specific epitopes or have only a few, yet when an antigen (protein) mixture is reformulated in accordance with the present invention so that the varieties of antigens (the various epitopes) are present in approximately equivalent amounts, maximum strength of the antigen compositions is achieved.

The specific antibody capture assay of the present invention employs a labeled reformulated antigen and reliably and reproducibly quenches, by a novel means, non-specific, antibody-based recognition. In addition, the reformulated antigen mixture of the present invention has a larger specific epitope repertoire than used in other methods. This large repertoire greatly increases assay sensitivity while maintaining high specificity of an assay for these compositions.

The antigenic components are not required to be "purified" as known in the art. Substantially all of an organism or allergen is used, where "substantially" means that no parts or fractions of parts are routinely or necessarily removed. The components are merely fractionated, and fractions pooled to enrich for proteins in smaller concentrations. Because of the ease of preparing the compositions of the present invention, rapid and reliable antigen adjustment is possible in case the strain of microorganism changes. An aspect of the invention is that the manufacturing process is standardized, resulting in batch to batch uniformity.

Antigen extraction and reformulation are achieved by the following steps:

1. extracting all soluble proteins from an antigen source;

2. fractionating the proteins;

3. mapping the fractionated proteins on SDS-PAGE;

4. determining relative amounts of individual proteins; and

5. combining sufficient and approximately equivalent amounts of the proteins into a composition. (The quantitative combining of fractionated proteins to achieve approximately equivalent amounts of each component in the combination is referred to as "reformulation").

More specifically, reformulating an antigen mixture is achieved by:

1. obtaining an antigen source, e.g. by culturing a microorganism of interest;

2. extracting all or a representative fraction of the constituent proteins of the antigen source;

3. fractionating the extracted proteins individually or in small groups;

4. determining the concentration of each protein within each fraction, e.g. by analyzing bands resulting from SDS-polyacrylamide gel electrophoresis;

5. combining a sufficient quantity of each protein either by adding individually purified proteins or by adding a predetermined quantity of a fractionated protein mixture, wherein the quantity of the desired protein or proteins within the mixture has been determined to afford an approximately equivalent end concentration of each constituent protein after all proteins originally extracted from the specific antigen source are remixed together, wherein constituent is defined as belonging to or derived from the target

organism/microorganism or allergen; and

6. labeling the reformulated antigen mixture.

The improved detection method of this invention is partially a result of reformulating the protein composition of the microorganism to be used in an assay so that all antigenic molecules or proteins of the microorganism are present in approximately equivalent amounts in the reformulation. The proteins in this reformulated composition are then labeled and used in the assays of this invention. Improvement is also due to the method by which non-specific epitopes are prevented from reducing assay accuracy.

In an embodiment, labeled, e.g. biotinylated, reformulated antigen mixture is spiked with non-biotinylated quenching elements, that is, non-labeled, non-specific epitopes. If the spiked antigen mixture is used, the captured immunoglobulins being held on the support bind protein molecules at both specific and non-specific epitopes. But because the non-specific epitopes are also found on the non-biotinylated (non-labeled) quenching proteins, and because the quenching proteins exist in far greater quantities within the antigen mixture than the corresponding labeled proteins, the quenching proteins preferentially bind to a subgroup of the captured immunoglobulin molecules and are unrecognized by analysis of the label. Conversely, those biotinylated molecules which possess specific epitopes are bound to the captured immunoglobulin molecules, and are recognized in later steps because they are labeled.

This invention provides a means for identifying recent or concurrent presence of immunoglobulins in a biological fluid which react with epitopes that

are specific or highly specific for an organism, as well as a method for measuring and quantifying the biological response to the organism in a subject. The biological fluid sample is selected from the group consisting of whole blood, plasma, serum, sputum, urine, cerebrospinal fluid, intra-abdominal fluid, intrathoracic fluid, pericardial fluid, joint space fluid, pustular fluid, tear fluid, nasal secretions, sinus fluid, and abscess fluid. Specific immunoglobulins that are detected by this method are IgA, IgD, IgE, IgG and IgM. Types of organisms and allergens suitable to practice the invention include bacteria, chlamydia, mycoplasma, protozoa, rickettsia, viruses, pollens, epidermal agents, mold spores, foods, venoms and allergenic pharmaceutical agents. *Helicobacter pylori* and *Chlamydia pneumoniae* are examples of specific suitable organisms from which compositions of the present invention are prepared. Small multicellular organisms such as intracorporal parasites also qualify as antigen sources for which there are many antigens and many epitopes per antigen. Helminths such as flukes (Trematodes), tapeworms (Cestodes), and roundworms (Nematodes) are examples of suitable parasites. Pollens comprise Orchard Grass Pollen, Brome Grass Pollen, Giant Ragweed Pollen, Pigweed Pollen, Smooth Alder Pollen and River Birch Pollen.

Individually, and in combination, each of the aspects of this invention increases the sensitivity and specificity of the assay for the presence of organism-specific antibodies in a biological fluid. Benefits of this new assay include detecting antibodies in the biological fluid of patients exhibiting symptomatology and/or diseases of infectious origin in order to determine the source of the

infection and the level of organism-specific immunoglobulin response in the patient's fluid sample. Ease of implementation and uniformity of antigen assays are other aspects of the invention.

A diagnostic kit useful for the practice of the present invention includes a capture antibody coated microtiter plate; and, in separate containers: a calibrator solution containing varying concentrations of analyte e.g. one with none and 4 with positive calibrator solutions, e.g. see Table 1; labeled (e.g. biotinylated) reformulated antigen mixture with unlabeled quenching proteins; streptavidin-alkaline phosphatase; 4-methylumbelliferyl phosphate; a wash buffer; a serum diluent; a conjugate diluent; and a substrate buffer.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagrammatic representation of anti-immunoglobulin antibody molecules (anti-Ig) coupled to a support, wherein the anti-immunoglobulin antibody molecules are capable of capturing antibodies from a sample of biological fluid antibodies that may carry both antigen specific (SBR) and non-specific epitope (NSBR) binding sites (receptors).

FIG. 2 is a diagrammatic representation of biological fluid sample antibodies captured by anti-immunoglobulin antibody molecules coupled to a support (FIG. 1). The captured antibodies depicted have either epitope-specific (SBR) or non-specific epitope binding sites (receptors) (NSBR).

FIG. 3 is a diagrammatic representation of an immunoassay system including labeled (*) antigen molecules obtained from a complex antigen source or organism such as a bacterium. The antigen molecule (diagonal lines) may

contain both specific (SE)(◊) and non-specific (NSE) (dotted square) epitopes, or all SE or all NSE. Mixed in with the labeled antigen molecules are antigen molecules from other organisms or other complex antigen sources (solid) which display non-specific epitopes similar to (homologous to) the non-specific epitopes on the labeled antigen molecules. Both specific and non-specific epitopes from a target organism can be bound by the immunoglobulin-anti-immunoglobulin complex attached to the support, said binding taking place at the specific epitope binding receptors (SBR) or the non-specific epitope binding receptors (NSBR) respectively.

FIG. 4 is a diagrammatic representation of an embodiment of the immunoassay of FIG. 3 in which an antigen molecule is labeled by biotin (*), and the means to detect the label is via streptavidin (SA)/alkaline phosphatase (AP) conjugated to the biotin and also indirectly to the antigen. S symbolizes the substrate which when processed by the labeling reactions, produces fluorescence (F) which is detectable by a fluorescent scanning device.

FIG. 5 is a graphical representation of the quantitative effects of various non-specific epitope sources (NSE) on detection of *H. pylori* specific serum IgG. The symbol □ refers to non-biotinylated reformulated *C. jejuni* antigen; to non-biotinylated reformulated Giant Ragweed antigen; O to non-biotinylated reformulated C. *jejuni* and Giant Ragweed antigens; and M to non-biotinylated reformulated *H. pylori* antigen.

FIG. 6 is a calibration (standard) curve derived by using sera with known quantities in "Enteron units" of IgG specific to *H. pylori*.

FIG. 7 is a graphical representation of joint levels of *H. pylori* (HP) specific serum IgG (Y-axis) and IgE (X-axis) in "Enteron units" in serum obtained from 96 subjects with HP related disease as determined by current standard methods (esophagogastroduodenoscopy and biopsy). All subjects showed evidence of *H. pylori*. The symbol M refers to subjects with duodenal ulcers, r to subjects with gastric ulcer, and F to subjects with chronic gastritis.

FIG. 8 is a graphical representation of HP specific serum IgG levels (Y-axis) and IgE levels (X-axis) in "Enteron units" in serum from 34 subjects without the diseases defined in FIG. 7, although some subjects have evidence of *H. pylori* infection based on IgG levels.

FIG. 9 is a graphical representation of the percent change of IgE times IgG level [reduction (-), increase (+), or no change (0) (Y-axis)] a number of days after cessation of anti-microbial therapy (X-axis) used in an attempt to eradicate *H. pylori*. M represent subjects in which no HP organisms were detected post-therapy by gastroscopy with biopsy or a urease assay; F represent subjects in which HP was still detected post-therapy.

FIG. 10 shows IgE changes in the same subjects shown in FIG. 9.

FIG. 11 shows IgG changes in the same subjects shown in FIG. 9.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates novel antigen compositions and means for dealing with the problem of non-specific epitopes reacting in an assay for a specific organism or allergen, thereby reducing accuracy of tests for specific target organisms or allergens. The compositions are used in the specific antibody

capture assay of the present invention which comprises the steps of (i) attaching an anti-immunoglobulin to a support, generally an insoluble support; (ii) capturing antibodies of a biological sample fluid by complexing with the anti-immunoglobulin antibodies (immunoglobulins) adhered to the support, said complexing is achieved by exposing a biological fluid sample to the support for a sufficient period of time to allow the isotype-specific antibodies within the biological fluid sample to complex with the specific anti-immunoglobulin molecules attached to the support, and then removing the uncomplexed fluid; (iii) exposing a mixture comprising labeled reformulated antigen molecules described herein, and nonlabeled reformulated or non-labeled non-reformulated antigen molecules containing non-specific epitopes homologous to the non-specific epitopes of the labeled reformulated antigen, to the support for a period of time sufficient to allow the antigen molecules to be bound by the matching type of the captured immunoglobulins; (iv) removing uncomplexed antigen molecules; and (v) detecting the presence of labeled antigen remaining on the support, from which the presence of antibodies in the biological fluid sample that are specific for the labeled antigen is determined, and the presence of the target organism or allergen is inferred. The amount of antigen-specific antibody in the biological fluid sample is determined.

FIGS. 1-4 illustrate a specific antibody capture assay. FIG. 1 depicts the anti-immunoglobulin antibody coupled to a support (immobilized, complexed). The anti-immunoglobulin antibody is capable of capturing both antigen-specific immunoglobulin with specific binding sites for antigens of the target organisms or

allergens and non-specific immunoglobulin with non-specific binding sites. A single isotype or several isotypes originating in a particular mammal are suitable, wherein immunoglobulin includes IgA, G, M, D, E antibodies. Several isotypes may be analyzed together if their binding to specific epitopes is distinguishable. Suitable anti-Ig includes a monoclonal antibody or affinity purified polyclonal antibody specific for any type of Ig, e.g. A, G, E, M, or D, or immunoglobulin binding proteins such as protein A and protein G. (Harlow and Lane, 1988) Each anti-Ig antibody or immunoglobulin binding protein binds at the Fc portion of the captured antibody from the biological fluid sample, leaving antibody Fab sites available for antigen binding.

FIG. 2 is a schematic presentation of the anti-Ig of FIG. 1 complexed with immunoglobulins that may have binding sites (receptors) specific for an epitope of an antigen being assayed, (SBR) and binding sites (receptors) that are present in, but non-specific for, the antigen of interest (NSBR). FIG. 2 depicts the binding (capture) of mammalian antibodies which occurs when a biological fluid sample containing antibodies capable of complexing with the anti-antibody molecule is added to the support. Biological fluids are generally obtained from a mammal, and include whole blood, plasma, serum, sputum, urine, cerebrospinal fluid, intra-abdominal fluid, intrathoracic fluid, pericardial fluid and other fluids found in various body cavities and spaces. FIG. 2 depicts a captured antibody capable of binding an organism-specific epitope (SBR) and another captured antibody capable of binding a non-specific epitope (NSBR).

FIG. 3 is a schematic presentation of the anti-Ig of FIG. 1 bound to a solid

support, complexed with immunoglobulin molecules in the biological fluid sample to be assayed, and also depicts the captured antibodies binding antigenic molecules either by their specific epitopes (SE) or their non-specific epitopes (NSE). Molecules may have epitopes specific for an organism or allergen that is the target of an assay, as well as non-specific epitopes. Molecules from sources other than the organism or allergen of interest are selected to contain non-specific epitopes that are the same in terms of binding abilities, as the non-specific epitopes on molecules from the target organisms or allergens. Shown both floating free and attached to the immunoglobulin binding sites are antigen molecules e.g. from *H. pylori*, with both specific (SE)(◊) and non-specific (NSE) (dotted square) epitopes. One labeled molecule is shown only with NSE. Unlabeled molecules with only non-specific epitopes are shown both floating free and attached to immunoglobulin binding sites. If the NSE on these molecules tie up the non-specific binding sites (NSBR), the NSE on the labeled molecules of the target organism will not be able to bind. Therefore, binding by the molecules of interest will be due to binding of the specific epitopes (SE). Because more non-specific epitopes are presented on unlabeled antigen molecules, the unlabeled antigens are preferentially captured by the antibody having NSBR. Labeled antigens which have no competition are bound via their specific epitopes to the captured antibody. The quantity of non-specific antigen required for successfully preventing the labeled, reformulated antigen from binding to captured antibody when the two are mixed together is 10 to 1,000-fold greater in quantity than the corresponding labeled antigen. A preferred embodiment is a factor of about 70:1

for reformulated non-specific epitopes.

FIG. 4 is a schematic presentation of elements shown in FIGS. 1-3 with the addition of the fluorescent labeling system fluorescent label (F) effected by biotin (*), streptavidin (SA), alkaline phosphatase (AP), and substrate(S). Other labeling systems are also suitable, such as radionuclides.

FIG. 5 is a graphical illustration of the quantitative effects of non-specific antibody sources (NSE) that are useful for "spiking." Shown are curves for three antigen sources: □ represents values for non-biotinylated, reformulated *C. jejuni* antigen; ◊ for non-biotinylated, reformulated Giant Ragweed antigen; ○ for non-biotinylated reformulated *C. jejuni* and Giant Ragweed antigens, and non-biotinylated, reformulated *H. pylori* antigen. The X-axis relates the ratio of spiking antigen to biotinylated reformulated *H. pylori* antigen.

The Y-axis depicts an *H. pylori* specific IgG sample with an initial FSU reading of 3300. As can be seen from the separate plots, the most distantly related antigen source, Giant Ragweed, reaches a plateau sooner than the more similar (more homologous) sources. This is because after the NSE in Ragweed occupy matching antibody sites, addition of more Ragweed antigen has no further quenching effects while more related sources to HP will tie up more NSBR because they are likely to share more epitopes with HP.

The values on the curve for C. *jejuni* and Giant Ragweed antigen is not the sum of the values for the two antigen sources because they share some non-specific epitopes that compete for some sites.

FIG. 6 illustrates calibration of the fluorescent measuring system of the capture antibody assay for *H. pylori* - specific serum IgG. The regression equation is shown in the upper left hand corner of the figure. Similar calibration is used for other Ig types. For IgE, undiluted ("neat") serum is generally suitable. Dilutions are preferred for IgG, A, D and M.

The steps of the invention are further defined as follows:

I. Attaching Anti-Immunoglobulins To A (Generally Insoluble) Support

An initial step in the assay of the present invention is adhering an anti-immunoglobulin to a support; preferably a support that is insoluble in any of the reagents used in the assay. Various types of insoluble surfaces provide a suitable support for the anti-immunoglobulin. Considerations in selecting the insoluble support are the ability of the support to bind the anti-immunoglobulin to the surface, the absence of interference with the labeled antigen, substrate and reagents, ease of use, and cost.

Organic and inorganic polymers, both natural and synthetic, are suitable as a support. Examples of suitable polymers include polyethylene, polypropylene, polybutylene, poly(4-methylbutylene), butyl rubber and other synthetic rubbers, silicone rubbers and silastic polymers, polyesters, polyamides, cellulose and cellulose derivatives (such as cellulose acetate, nitrocellulose and the like), acrylates, methacrylates, vinyl polymers (such as polyvinyl acetate, polyvinyl chloride, polyvinylidene chloride, polyvinyl fluoride, and the like), polystyrene and styrene graft copolymers, styrene-acrylonitrile copolymers, rayon, nylon,

polyvinylbutyrate, polyformaldehyde, and so forth. Other materials which are suitable as a support are silica gel, silicon wafers, glass, paper, insoluble proteins, metals, metaloids, metal oxides, magnetic materials, semi-conductive materials, cements or the like. In addition are included substances that form gels, such as proteins, gelatins, lipopolysaccharides, silicates, agarose, polyacrylamides, polymers, or polysaccharides which form several aqueous phases such as dextrans, polyalkylene glycols (alkylenes with 2 to 3 carbon atoms) or surfactants, e.g. amphophilic compounds such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like.

Preferred supports of this invention comprise a polystyrene, styrene copolymers including styrene-(vinyl monomer) copolymers such as styrene-acrylonitrile copolymers, or polyolefins such as polyethylene and polypropylene, and acrylate and methacrylate polymers and copolymers. The capture antibody or antiserum is bound thereto by adsorption, ionic bonding, van der Waals adsorption, electrostatic bonding, other non-covalent bonding. The antibody can also be bound to the support by covalent bonding. A particularly advantageous support for this invention comprises a polystyrene microtiter plate having a plurality of wells. The well surface or plastic cup inserts therein can constitute the antibody support. Most advantageously, the microtiter plate or the well inserts are opaque to light so that excitation light applied to a well or fluorescence generated in response thereto does not reach or influence contents of the surrounding wells. With this system each well can be employed as a test system independent of the other wells.

II. Capturing Test Fluid Antibodies by the Anti-Immunoglobulin Antibody Adhered to the Support

After coupling the anti-immunoglobulin to the insoluble support, e.g. a microtiter plate well, the test subject's biological fluid sample (test fluid) is exposed to the insoluble support. Table 1 shows an example of the layout of a microtiter plate wherein the numbers in each square refer to samples, and "Calib." and "Neg.cont." are internal standards.

Each test fluid is diluted with an appropriate immunoglobulin Assay Diluent. The degree of dilution depends upon the total quantity of the type of immunoglobulin sought within the biological fluid sample. In the microtiter plate format described in part I herein, it is usually important not to have more antibody in the sample than can be captured by the capture antibody or capture antisera adhered to the microtiter well or other solid surface (approximately 0.2μg per 100μL well sample). For human serum IgA, IgD, IgG, and IgM, an appropriate assay serum diluent is 10 mM Tris-HCl, pH 7.5, containing 600 mM NaCl, 4.0 mg/mL casein, 30.0 mg/mL PEG-4000 (polyethyleneglycol 4000) and 0.2 mg/mL thimerosal. For IgE, an appropriate assay serum diluent is 14.3 mM Tris-HCl, pH 7.5, containing 214.5 mM NaCl, 5.72 mg/mL casein, 42.9 mg/mL PEG-4000 and 0.28 mg/mL thimerosal, although a preferred embodiment calls for using neat serum. Other formulations are suitable for specific applications. The amount of dilution varies depending on the immunoglobulin being targeted. For IgA, a dilution factor for the test serum (fluid) is about 2,200-fold. For IgD, a dilution factor for the test serum (fluid) is about 90-fold. For IgE, a dilution factor for the

test serum (fluid) is about zero to 4-fold. For IgG, a dilution factor for the test serum is 10,000-fold, with a range of 0-10,000 fold. For IgM, a dilution factor for the test fluid is about 1500-fold.

The microtiter plate is washed 3 times with 300µL of Immunoassay Wash Buffer per well (the Wash Buffer is 10 mM Tris-HCl, pH 7.5, containing 600 mM NaCl, 2.0 mg/mL casein and 0.2 mg/mL thimerosal). 100µL of each diluted or neat test serum is added to the appropriate wells of the plate. The monoclonal or affinity-purified polyclonal anti-immunoglobulin coupled to well surfaces captures all or a representative amount of total targeted immunoglobulin in the test sample. In the microtiter plate embodiment described, about 0.2µg of capture antibody per well provided the best results. The plate is covered and incubated at 25°C for between 5 minutes-24 hours; preferably between 2 to 18 hours, and more preferably about 4 hours. Then, the biological fluid sample is aspirated and the microtiter plate is washed five times with the Immunoassay Wash Buffer.

III. Exposing Labeled Reformulated Antigen And Non-Labeled Non-Specific Antigen To The Captured Antibody On The Support

Labeled reformulated antigen and nonlabeled, non-specific antigen which share homologous epitopes with the labeled reformulated antigen, are added to the microtiter plate wells. Preferably labeled and non-labeled antigen are mixed prior to being added to the wells. The epitope-specific capture sites of the bound isotype-specific antibodies bind with the labeled reformulated antigen-specific epitopes, whereas the non-specific epitope capture sites bind with the non-specific unlabeled antigen epitopes.

A. Preparing the Reformulated Antigen Composition

An important and novel aspect of this invention is the use of a composition which is an antigen mixture which has been reformulated so that all antigenic molecules are equally represented in the antigen mixture. The preferred method for making this reformulated antigen mixture involves obtaining (purchasing or culturing) a specific organism, microorganism or microorganisms or parasite, or allergen. All or a substantial fraction of soluble proteins are extracted from the organism or allergen. The extracted proteins are then separated from the initial mixture into smaller groups by fractionation based on their solubility, size, ionic charge or other chemical characteristics. Finally, the fractionated proteins are reformulated into a new mixture where the quantitative presence of each protein is approximately equivalent to that of the other proteins in the mixture. The resulting reformulated antigen mixture is labeled and used in the isotype-specific antibody assays described herein. Preferably, the reformulated antigen mixture is combined with the quenching proteins (non-specific antigen) described herein and used in the assay of this invention. The non-specific antigen may be reformulated by the same procedures from a different antigen source than the target organism or allergen.

B. Labeling the Reformulated Antigen from the Target Organism or Allergen

There are many suitable labels to attach to the reformulated antigens from a target organism. A preferred method of labeling reformulated antigen is biotinylation. However, the proteins can also be labeled with, by way of example,

enzymes, fluorophores, chromophores, and radionuclides.

Biotinylation is accomplished by selecting the appropriate quantity of concentrated reformulated antigen extract and reacting with NHS-LC-biotin. A 2.5 x 16 cm Sephadex G-15 column is equilibrated with at least 800 mL Biotinylated Antigen Storage Buffer (consisting of 10 mM Tris-HCl, pH 8.0 + 0.1% SDS + 0.02% NaN3 + 1 mM EDTA + 1 mM EGTA) at a flow rate of approximately 10 mL/min. Reformulated antigen extract is centrifuged at 100,000 x g for 30 minutes at 4°C. The supernatant is collected, the pellet(s) is discarded, and the protein concentration of the collected supernatant is determined using the Lowry protein assay. (Waterborg and Matthews, 1994)

The amount of NHS-LC-biotin required for the reaction is calculated. Molar ratios of 5:1 to 70:1 biotin to protein are suitable, but a ratio of approximately 20:1 is preferred. The average molecular weight of the protein mixture is calculated by densitometry studies of PAGE gels containing the protein mixture separated into individual protein bands (Peck et al., 1988). For example, the average estimated molecular weight for *Helicobacter pylori* proteins in the reformulated protein mixture is about 60,000.

The pH of the antigen extract supernatant is adjusted to 8.70±0.10 with 0.1 M sodium hydroxide solution. The calculated amount of NHS-LC-biotin is added to the antigen extract supernatant. The mixture is capped tightly and vortexed immediately. The reaction mixture is placed on a rotating mixer and incubated at ambient temperature for 45 minutes. At the end of the 45 minute reaction time, solid Tris base is added to the reaction mixture to a final concentration of 0.5 M

(60 mg Tris base per mL biotinylated antigen solution). The container is capped tightly and vortexed immediately. The reaction mixture is placed on a rotating mixer and incubated at ambient temperature for 10 minutes.

The reaction mixture is applied to the equilibrated Sephadex G-15 column at a flow rate no greater than 5 mL/min, collecting fractions of approximately 4 mL. The OD280 of each fraction is determined and the elution profile is plotted. The biotinylated antigen-containing fractions are pooled into a suitably sized graduated cylinder. (These comprise the protein-positive, e.g. the OD280-positive, fractions.) The protein concentration of the pool is determined using the Lowry protein assay. The biotinylated antigen mixture is then aliquoted and a sufficient quantity is added to the support to bind with the anti-immunoglobulin-immunoglobulin complex. Alternatively, the labeled reformulated antigen can be frozen at -20°C or lower for later use.

C. Applying the Labeled Reformulated Antigen To the Support

To apply the labeled reformulated antigen to the support, wherein the support is a microtiter plate, 100µL of Biotinylated Antigen Mixture is added to each test well on the plate, covered and incubated at 25°C for 75 minutes. For IgA, IgD, IgG and IgM antibodies, the Immunoassay Antigen Mixture suitably comprises about 1-4µg/mL biotinylated reformulated antigen, with or without quenching proteins, in a solution comprising 10 mM Tris-HCl, pH 7.5, 600 mM NaCl, 4.0 mg/mL casein, 30.0 mg/mL PEG-4000 and 0.2 mg/mL thimerosal. For IgE, the Immunoassay Antigen Mixture suitably comprises 2-10µg/mL biotinylated reformulated antigen, with or without quenching proteins, in a

solution comprising 10 mM Tris-HCl, pH 7.5, 600 mM NaCl, 4.0 mg/mL casein, 30.0 mg/mL PEG-4000 and 0.2 mg/mL thimerosal. The labeled antigen solution is incubated for a time ranging from 2 minutes to 24 hours. A range of 2 to 180 minutes is suitable. In a preferred embodiment of this invention the incubation time is 75 minutes.

When using a reformulated antigen mixture from a target organism with quenching ingredients (generally proteins), some of the captured antibodies being held on the support bind antigen molecules at their specific epitopes and others bind antigens at their non-specific epitopes. Because the non-specific epitopes are also found on non-biotinylated (unlabeled) quenching antigens, and the quenching antigens exist in far greater quantity within the complex mixture than do the labeled antigens, the non-labeled antigens preferentially bind to the captured immunoglobulin molecules which have affinity for the non-specific epitopes. The bound quenching antigens go unrecognized because they are not labeled. The biotinylated reformulated antigens which possesses specific epitopes bind to other captured immunoglobulin subgroups and are recognized in later steps because they are labeled.

The microtiter plate is washed five times with the Immunoassay Wash Buffer (comprising 10 mM Tris-HCl, pH 7.5, containing 600 mM NaCl, 2.0 mg/ml casein and 0.2 mg/mL thimerosal). 100µL of a diluted streptavidin/alkaline phosphatase conjugate mixture is added to each well. The streptavidin/alkaline phosphatase binds to the available biotin. For IgA, IgD, IgG, and IgM antibodies, the conjugate mixture comprises 0.75 ng/mL

streptavidin/alkaline phosphatase conjugate, in a solution comprising 10 mM Tris-HCl, pH 7.5, 600 mM NaCl, 4.0 mg/mL casein, 30.0 mg/mL PEG-4000 and 0.2 mg/mL thimerosal. About 75 ng of streptavidin/alkaline phosphatase conjugate is added per well. For IgE, the conjugate mixture comprises 2.75 ng/mL streptavidin/alkaline phosphatase conjugate, in a solution comprising 10 mM Tris-HCl, pH 7.5, 600 mM NaCl, 4.0 mg/mL casein, 30.0 mg/mL PEG-4000 and 0.2 mg/mL thimerosal. About 275 ng of streptavidin/alkaline phosphatase conjugate is added to each well. The plate is covered and incubated at 25°C for 5 to 60 minutes. A preferred embodiment is 15 minutes. The microtiter plate is washed six times with an Immunoassay Wash Buffer.

IV. Adding The Non-Specific Antigens

Another aspect of this invention is the use of non-specific antigens to quench non-specific epitopes being bound by the captured, biological fluid immunoglobulin. The molecules with non-specific antigens to be added are non-labeled and are selected to have epitopes in common with (that is, homologous to) non-specific epitopes on the specific antigen(s) of interest. Homologous is defined herein to mean alike or corresponding for characteristics relevant to the present invention, e.g. derived from a single or taxonomically related individual so that antigenic composition is similar, that is, there are some shared epitopes that can compete successfully for binding sites on antibodies.

Choice of non-specific epitope depends on the antigen source. For example, the non-specific epitope mixture ("sister proteins" or "matching proteins") is determined for microorganisms e.g. by using taxonomic relationships

to determine the best "quenching" in a known assay with appropriate controls. The purpose of the non-labeled proteins is to block the binding of the non-specific epitopes on the labeled antigens of interest. Antibodies in a subject's serum or other biological fluid which could bind to these homologous epitopes and thereby effect a non-specific recognition, instead bind to the corresponding non-specific epitopes on the nonlabeled proteins and are thereby not recognized during the assay procedure. Therefore, it is preferred that an antigen source for these proteins is related to the antigen source for the labeled reformulated proteins, although homologous molecular relationships can exist among sources of antigen which have distant taxonomic relationships one to the other.

The most direct way to extract proteins from the related or distant antigen source is in the same manner described for reformulating the target antigen described herein. Nonlabeled proteins can then be used to quench the non-specific, antibody-based recognition of the specific microorganism. The quantity of quenching proteins added to the assay varies depending on differences in antigen composition between individual organisms or other complex antigen sources. Antigen inhibition studies are the best way of determining how much spiking or quenching unlabeled antigen must be added to the labeled antigen. A suitable amount of spiking antigen(s) is that which provides maximum inhibition of non-specific epitopes. A gradual drop in the assay signal is observed until no more reduction is possible. The quantity of spiking antigen which gives the lowest reduction possible is that which is added to the labeled antigen. FIG. 5 shows examples of antigen inhibition for *H. pylori*.

V. **Reading The Result**

The final step of this assay is detecting the binding of labeled reformulated antigen to the complex on the support, from which the presence of antibodies in the biological fluid tested that are specific for the antigen, is inferred. If biotinylated antigen and streptavidin-alkaline phosphatase has been used, 100μL 4-methylumbelliferyl phosphate solution is added to each well, and the plate is read in a fluorescence microtiter plate reader, using 365 nm excitation and 450 nm emission, at 15 minute intervals for 1 hour. Standard curves are drawn using the test results of calibrator reagents which are run in parallel with the test sera or other biological fluid test samples. The unknown test results are plotted onto the standard curve, thereby ascertaining individual test values. (FIG. 6 represents a calibration curve for *H. pylori* IgG.)

If labels other than biotin are added to the reformulated antigen mixture, then the steps for identifying and reading the labels or markers are modified as appropriate and are known to those of skill in the art. These other labeling methods are adequately described in Tijssen (1985) and can be used as an alternative labeling method.

EXAMPLES

The following examples illustrate the method of extracting and reformulating proteins from organisms such as microorganisms, parasites, allergens and various other antigen sources. Those of ordinary skill in the art would recognize that these examples can be modified to make and reformulate proteins from other organisms and antigen sources. They would also recognize

that other methods can be used to reformulate the protein composition of an organism so that all antigenic molecules are present in approximately equivalent amounts.

Example 1: *Helicobacter pylori* Antigen Extraction and Reformulation Procedure

The first step requires obtaining the source for the antigen. Generally, *Helicobacter pylori* organisms are cultured to a desired quantity, generally about 150g. Techniques for culturing varying quantities of *H. pylori* are described by Deshpande, et al. (1995). Organisms are washed and concentrated using cold normal saline and a tangential flow concentrator. Organisms are then pelleted by centrifugation and frozen until needed.

To extract the protein from the *H. pylori*, the desired quantity of frozen *H. pylori* is added to an appropriately-sized, heat resistant container or beaker. For each gram (wet weight) of frozen *Helicobacter pylori*, 10 mL of a solution comprising 20 mM sodium phosphate buffer, pH 7.35, with 100 mg/mL octyl-beta-D-glucopyranoside (or other functional detergent) is added. The beaker is placed on a stirring hot plate, a stir bar is added and the mixture is stirred, and suspension is slowly warmed at approximately 1°C per minute, to 25°C. The heating element is then turned off but stirring continues while clumps of organisms are broken up by using a handblender for 90±10 seconds, until a uniform suspension is achieved. The suspension is stirred for 1 hour at ambient temperature. The homogenate is centrifuged at 100,000 rpm for 30 minutes at 4°C. The supernatant is collected and the pellet(s) are discarded. If multiple

centrifuge runs are required, the collected supernatant is held at 4°C and stirring continues for the remaining suspension at ambient temperature. After centrifuging is completed, the total supernatant volume collected, measured and recorded.

A number of dialysis bags is prepared, using 3500 MWCO dialysis tubing, which is sufficient to dialyze the accumulated supernatant. The *H. pylori* extract is dialyzed against 20 mM sodium phosphate buffer, pH 7.35, at 4°C, using a dialysate to bag volume ratio of at least 13:1, or no more than 300 mL bag volume in 4 L of dialysate. The dialysate is changed four times allowing at least 4 hours between changes. The dialyzed *H. pylori* antigen extract is collected in a suitably sized graduated cylinder, and the volume is recorded.

The next step involves fractional acetone precipitation of the antigen extracts. This step begins by measuring the tare weight of an appropriate number of centrifuge bottles with caps. The weight is recorded on each bottle. The dialyzed *H. pylori* antigen extract is transferred to a suitably sized screw capped glass container; a stir bar is added and the mixture is stirred.

The amount of acetone to be added to yield a concentration of 20 % acetone is calculated. The calculated amount of acetone is measured and added to the *H. pylori* antigen extract. The mixture is capped tightly and stirred at 4°C for 15 minutes. An appropriate number of weighed centrifuge bottles is labeled with the acetone percentage and an identification number, i.e. 20 % - 1, 20 % - 2, and so forth. The *H. pylori* antigen extract is transferred to the labeled centrifuge bottles. The *H. pylori* antigen extract is centrifuged at 6800 rpm at 4°C for 30 minutes. The volume of the combined supernatants is collected and measured (if

more than one container is used to centrifuge the acetone-supernatant suspension). The pellets are held at 4°C. The steps of calculating the acetone and measuring it into labeled centrifuge bottles are repeated, incrementally increasing the acetone concentration as follows: 25, 30, 35, 40, 45, 50, 55, 60, 65, and 80 percent. The 80% acetone supernatant is discarded. All of the centrifuge bottles are transferred to a chemical fume hood, the caps are removed and the residual acetone is allowed to evaporate at ambient temperature overnight.

Two 7.5% and two 12.5% SDS-polyacrylamide gels are prepared (Cooper, 1977).

The proteins found in the different acetone-precipitated fractions are resolubilized and the constituent proteins in each fraction are identified and quantified by SDS-Polyacrylamide Gel Electrophoresis (PAGE). Each centrifuge bottle is capped and weighed. The net weight of each pellet and the volume of 20 mM sodium phosphate buffer, pH 7.35 required for reconstitution at a ratio of 20 mL buffer per gram of pellet are calculated. The calculated volume of 20 mM sodium phosphate, pH 7.35 is added to each bottle. The capped centrifuge bottles are placed on an orbital mixer and shaken vigorously at 4°C for 30 minutes. The individual suspensions resulting from each acetone percentage are pooled into a single centrifuge bottle. The capped centrifuge bottles are placed on an orbital mixer and shaken vigorously at 4°C for 90 minutes.

Stacking gels for each of the previously prepared SDS-polyacrylamide gels are cast and allowed to polymerize for at least 1 hour.

Each of the reconstituted solutions are centrifuged to remove any

undissolved residue at 100,000 x g for 30 minutes at 4°C, in capped ultracentrifuge tubes. For volumes greater than 240 mL, centrifuging is at 6800 x g for 90 minutes at 4°C in capped bottles.

After centrifuging, the volume of the supernatant from each reconstituted solution is measured and recorded. The supernatants are held at 4°C. The pellets are discarded. The protein concentration of each supernatant is determined using the Lowry protein assay.

An appropriate volume of each supernatant is prepared for SDS-polyacrylamide gel electrophoresis by boiling the supernatant in sample buffer containing 2-mercaptoethanol. A 10 to 50μL aliquot of each treated protein solution, containing approximately 50μg of protein, is applied to individual lanes of both 7.5% and a 12.5% SDS-polyacrylamide gels. Molecular weight standards are added and electrophoresis is performed. The gels are stained and dried.

The final steps involve pooling and concentrating the proteins. Using gel densitometry, the approximate protein concentration is determined for each visible protein band seen on the SDS gel lane corresponding to each supernatant attained through the process described above. A sufficient volume from each supernatant is mixed to achieve approximately equivalent concentration of each of the different proteins, visualized in the SDS gels, in the resulting protein mixture. [Supernatants giving sharp clear bands on SDS-polyacrylamide gels are included while supernatants giving diffuse, blurred bands (generally 35 to 45 % acetone-cut fractions) are generally excluded from the pool.] A 5000 MWCO tangential flow concentrator unit is equilibrated with 20 mM sodium phosphate buffer, pH 7.35,

and the pool of reconstituted supernatants is concentrated using the equilibrated concentrator unit until a protein concentration of 15-30 mg/mL is attained. The protein concentration of the concentrate is determined using the Lowry protein assay. The concentrated *H. pylori* protein mixture can be frozen until needed.

Example 2: *Campylobacter Jejuni* Antigen Extraction And Reformulation Procedure

The method of making the reformulated *H. pylori* antigen mixture of Example 1 can be used to make a reformulated *Campylobacter jejuni* antigen mixture. A method for culturing *C. jejuni* organisms to the quantity desired for this extraction and reformulation procedure is described by Rollins et al. (1983).

Example 3: Orchard Grass Pollen Antigen Extraction And Reformulation Procedure

Defatted Orchard Grass Pollen (raw antigen) used in this extraction and reformulation procedure can be purchased from a variety of reliable sources, such as Crystal Laboratories in Luther, Oklahoma. A sufficient quantity of raw allergen is transferred to a suitably sized screw cap container containing of 50 mM sodium phosphate buffer, pH 8.0 or other suitable buffer. The ratio of buffer to defatted pollen is 10 mL buffer to 1 gram dry weight pollen. The container is securely attached to an orbital mixer and the suspension is agitated vigorously for 48 hours at 4°C. Upon completion, the resulting suspension of crude extract is transferred to a suitable centrifuge bottle(s), and the crude extract is centrifuged at 6500-6800 x g for 1 hour at 4°C. The supernatant is transferred to a suitably sized graduated cylinder. A small amount of pellet material may be included with the supernatant. The pellet is discarded and the supernatant volume is recorded.

The supernatant is transferred to dialysis bags prepared from pretreated 45 mm 3500 MW cutoff dialysis tubing. The supernatant is dialyzed against 50 mM sodium phosphate buffer, pH 8.0, at 4°C at a ratio of not less than 20 mL of dialysate per mL supernatant. The dialysate is changed four times allowing a minimum of four hours between changes.

The dialyzed supernatant is transferred to a suitably sized graduated cylinder so that it can be easily transferred to a suitable number of ultracentrifuge tubes. The supernatant is centrifuged in the tubes at 100,000 x g for 1 hour at 4°C. If multiple runs are required, the unused portion of the dialyzed supernatant is held at 4°C. After each run, the supernatant is collected in a suitably sized graduated container and the pellet is discarded. The collected supernatant should be free of pellet residue. If the pellet is disturbed during supernatant collection, the tube should be centrifuged again. The collected supernatant is held at 4°C until all ultracentrifuge runs are completed. The total supernatant volume is recorded.

A 5000 MWCO tangential flow concentrator cassette is flushed with purified water. The cassette is equilibrated with 50 mM sodium phosphate buffer, pH 8.0. The ultracentrifuged supernatant collected above is diluted to a concentration of 25-30 mg/mL. This concentrate may be frozen until needed or it can be frozen after fully processed.

The next step involves fractional acetone precipitation of the antigen extracts and can be performed in the same manner as described in Example 1 for fractional acetone precipitating of the *H. pylori* antigen extracts, and therefore, is not repeated here.

After fractional acetone precipitation of the Orchard Grass pollen antigen extracts, the proteins are resolubilized in different acetone-precipitated fractions and the protein constituents in each fraction are determined by SDS-Polyacrylamide Gel Electrophoresis (PAGE). These steps can be performed in the same manner as described in Example 1, and therefore, are not repeated here.

The final steps involving pooling and concentrating the proteins. Using gel densitometry, the approximate protein concentration of each visible protein band seen on the SDS gel lane corresponding to each supernatant attained through the work described above is determined. A sufficient volume from each supernatant is mixed so as to achieve equivalent concentrations of each of the different proteins visualized in the SDS gels in the resulting protein mixture. A 5000 MWCO tangential flow concentrator unit is equilibrated with 20 mM sodium phosphate buffer, pH 7.35. With equilibration buffer circulating in the concentrator unit, the system pressure is adjusted to 40 psi. The pool of reconstituted supernatants is concentrated using the equilibrated concentrator unit until a protein concentration of 25-30 mg/mL is attained. The protein concentration of the concentrate is determined using the Lowry protein assay. After preparing this concentrated Orchard Grass Pollen protein mixture, it can be frozen until needed.

Example 4: Extraction and Reformulation Procedure For Other Allergen Source Proteins

The procedure described in Example 3 for extracting and reformulating the proteins in Orchard Grass Pollen works equally well with other allergen sources,

such a Brome Grass Pollen, Giant Ragweed Pollen, Pigweed Pollen, Smooth Alder Pollen, and River Birch Pollen, and is suitable to reformulate the proteins of these allergens.

Example 5: Composing a Nonlabeled Antigen To Quench Nonspecific Epitope Binding On Labeled *H. pylori* Antigen

An embodiment of the method of combining nonspecific antigens with the labeled reformulated *H. pylori* antigen to make a antigen solution capable of quenching nonspecific binding on the labeled antigen is as follows. Those of ordinary skill in the art would recognize that this example can be modified to make mixtures that would be effective in quenching nonspecific binding of other labeled proteins. They would also recognize that other methods can be used to make this mixture of labeled specific antigen and nonlabeled nonspecific related antigen which has homologous epitopes to those nonspecific epitopes on the labeled antigen.

To compose a reformulated antigen mixture, where non-biotinylated nonspecific proteins serve to block out or quench non-specific epitopes on *H. pylori* proteins, add 70 mg of reformulated *Campylobacter jejuni* proteins and 30 mg of each reconstituted allergen mixture (comprising Orchard Grass Pollen, Brome Grass Pollen, Giant Ragweed Pollen, Pigweed Pollen, Smooth Alder Pollen, and River Birch Pollen reformulated proteins) to each mg of reformulated biotinylated *H. pylori* protein mixture. This combination can then be used effectively in the assay of this invention.

Example 6: Using the Assay of the Present Invention to Detect Conditions Associated With *H. pylori*

The methods of the present invention were used to detect and to monitor the course of diseases and conditions associated with *H. pylori*. The methods proved particularly useful for monitoring the effects of anti-microbial therapy, and the course of diseases associated with *H. pylori*.

Current methods of diagnosis, esophagiogastroduodenoscopy (EGD) and biopsy were used on subjects. Some subjects were found to have chronic gastroduodenal disease, e.g. gastric ulcers, duodenal ulcers, gastritis, duodenitis, esophagitis. Some subjects had more than one condition.

Among those subjects who tested positive for the disease, some were infected with *H. pylori* as determined by histochemical tests and/or the urea breath test. Other subjects did not evidence *H. pylori* infection.

Serologic testing was done to determine whether a subject had organism-mediated disease. There are two parts to a complete answer to this question:

1. Is the subject infected with *H. pylori*?

2. If the subject is infected with *H. pylori*, is the infection causing a disease?

Detection of IgG specific for *H. pylori* (HP) in a subject is indicative of HP infection. Detection of IgE specific for *H. pylori* (HP) is indicative of the presence of HP associated disease in a subject. (Calenoff, 1996) An aspect of the present invention is that it is information on a combination of both IgG and IgE levels that provides more accurate determination of organism-specific disease than

is provided by information on the level of either immunoglobulin alone.

FIG. 7 is a graphical representation of serum levels of HP-specific IgG (Y-axis) and IgE (X-axis) in "Enteron units" an arbitrary unit; serum is from 96 subjects with HP related disease.

FIG. 8 is a graphical representation of HP specific-serum IgG levels (Y-axis) and IgE levels (X-axis) in "Enteron units"; serum is from 34 subjects without HP-related diseases. The subjects forming the basis of FIG. 8 were also without evidence of *H. pylori* organisms from standard tests, but are shown to the carriers of HP by elevated specific IgG levels.

Example 7: Using the Assay of the Present Invention to Monitor the Effect of Treatment on *H. pylori* Eradication

Subjects who had evidence of *H. pylori* infection and disease were treated with standard antibiotic therapy to eradicate the organism, thereby alleviating the disease. Using the methods and compositions of the present invention, levels of IgG and IgE specific for HP were determined immediately prior to initiation of treatment, and at various times after treatment.

FIG. 9 is a graphical representation of the percent change of IgE times IgG level reduction or increase (Y-axis) days after cessation of anti-microbial therapy (X-axis) used in an attempt to eradicate *H. pylori*. (Antibiotics and acid lowering agents are used.) ● represents subjects in which no HP organisms were detected post-therapy by gastroscopy with biopsy; O represent subjects in which HP was still detected post-therapy.

FIGS. 10 and 11 show the IgE and IgG values respectively for the same

subjects shown in FIG. 9. The conclusion from a comparison of FIGS. 9, 10 and 11 is that the value of (IgE X IgG) presents a clearer delineation of effects of antimicrobial treatment at various times after cessation of therapy. The first reading was taken before administering treatment; the second at various times post treatment as shown. A limitation of using IgG values alone is that they do not seem to fall as fast as IgE levels in the same patients. The combined formula is a more useful measure for monitoring the effects of treatment.

Example 8:A Diagnostic Kit

A diagnostic kit includes a support and, in separate containers, calibrator solutions and a labeled reformulated antigen mixture containing quenching antigens. Table 1 shows an example of a support with a matrix of wells (a microtiter plate) with calibration solutions and a negative control. Sample numbers are indicated in each well. An example of a set of calibrator solutions is as follows:

Calibrator	IgG	IgE
a	1000 U/ml	125 U/ml
b	500 U/ml	62.5 U/ml
c	250 U/ml	31.25 U/ml
d	125 U/ml	16.125 U/ml
Negative Control	0 U/ml	0 U/ml

The support is scanned for presence of label in each well; e.g. if a fluorescent label was used, a fluorescence scanning device is used.

Example 9: Using the Assay of the Present Invention to Detect Conditions Associated with *Chlamydia pneumoniae*

Antigens were extracted and reformulated from *Chlamydia pneumoniae* by the methods of the present invention. These are essentially similar to the methods of Example 1. The reformulated mixtures were used to detect conditions associated with the presence of *Chlamydia*. A particular condition associated with *Chlamydia* is accelerated atherosclerotic plaque formation. IgE and IgG levels were measured and multiplied. Subjects with the condition were differentiated from normal using this value.

MATERIALS AND METHODS

I. Ultraviolet Light Activation and Covalent Coupling Procedure

A procedure to covalently attach various proteins to polystyrene microtiter plates is as follows:

Materials

Polystyrene Microtiter Plates, for example: Dynatech Microtite 1, cat. no. 011-010-7416; Dynatech Immulon 4, cat. no. 011-010-3855; or equivalent.

Succinic anhydride, Sigma cat. no. S-7626 or equivalent.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, Sigma cat. no. E-11769 or equivalent.

A protein to be covalently coupled, for example: A purified monoclonal antibody; a purified polyclonal antibody; a protein with a specific binding capacity such as NeutrAvidin (Pierce cat. no. 31000); a purified antigen for an antibody of choice.

Buffers and solutions

A. 50 mM sodium borate, pH 8.10 ± 0.05

B. 50 mM sodium borate, pH 8.80 ± 0.05

C. 10 mM sodium phosphate, pH 7.20 ± 0.05

5M sodium hydroxide

100 g/L Sucrose with 200 mg/L sodium azide

Equipment

Ultraviolet (UV) light box, Fotodyne model 3-3000, or equivalent, equipped with bulbs delivering light at 254 nm.

Multichannel pipettors

Procedure

1. Place a suitable number of polystyrene microtiter plates face down on a UV light box. Expose the plates to the 254 nm UV light for 40 minutes. (The exposure period may require optimization for specific applications.) During the activation time the plates may be rotated or repositioned occasionally to equalize the exposure of the internal surfaces of the wells of the light.

2. Approximately 15 minutes before the end of the UV exposure time, prepare a 5 mg/mL solution of succinic anhydride in Buffer A. At the end of the UV activation period, adjust the pH of the succinic anhydride solution to 8.40 ± 0.20 with 5M sodium hydroxide. Immediately add 200µL succinic anhydride solution to each well of the activated microtiter plates. Fresh succinic anhydride solution must be prepared for each set of activated plates.

3. Incubate the succinic anhydride solution in the wells for 10 minutes.

4. Wash the wells three times with deionized water. If multiple sets of plates are being prepared, the succinylated wells may be held in deionized water at ambient temperature.

5. Prepare a fresh solution of the protein to be coupled in Buffer B at the desired protein concentration. For example, a 10µg/mL solution of a monoclonal antibody and a coating volume (step 8) of 100µL per well would yield 1µg of protein for coupling in each well. The coupling protein concentration must be optimized for each application.

6. Prepare a fresh solution containing 100 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in Buffer C.

7. Aspirate the deionized water from the succinylated microtiter plate wells. Add 110µL EDC solution to each well. Incubate at ambient temperature for 10 minutes.

8. Aspirate the EDC solution from the wells and immediately add

100μL of the coupling protein solution (step 5) to each well. Cover the plates and incubate at ambient temperature overnight.

9. Aspirate the protein solution from the wells. Add 300μL sucrose solution to each well. Incubate at ambient temperature for 20 minutes.

10. Aspirate the sucrose solution and transfer the plates to a laminar flow hood to dry completely, approximately 4 hours.

11. Cover the dried plates and store at 4°C.

II. Antigen Biotinylation Procedure

The following extraction procedure is used to prepare biotinylated antigens.

Material

Antigen extract.

Sulfosuccinimidyl-6-(biotinamido)hexanoate, Pierce cat. no. 21335 or equivalent

2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS base)

Lowry protein assay reagents

Buffers

A. 50 mM sodium phosphate, pH 8.0 ± 0.20 with 150 mM sodium chloride and 1 mg/mL sodium dodecylsulfate

B. 50 mM sodium phosphate, pH 6.6 ± 0.1 with 150 mM sodium chloride, 1 mg/mL sodium dodecylsulfate, and 200 mg/L sodium azide

Equipment

Sephadex G-15 or G-25 desalting column

Vortex mixer

Tip plate or orbital mixer

Spectrophotometer

Procedure

Note: The antigen to be biotinylated must be dissolved in Buffer A at a suitable concentration, generally 10 to 30 mg/mL. If the antigen is not in Buffer A, perform a buffer exchange by dialysis or other suitable means.

1. Determine the protein concentration of the antigen to be biotinylated. The Lowry method using the BioRad Dc assay, cat. no. 500-0116, is recommended.

2. If required, concentrate the antigen using either a tangential flow or a pressure cell system equipped with a suitable membrane.

3. Equilibrate a suitably sized desalting column in Buffer B using at least 5 column volumes of buffer.

4. Calculate the amount of Sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin) required for the reaction. A 5 to 70 fold molar excess of NHS-LC-biotin over antigen is generally used. The appropriate ratio must be optimized for each application.

5. Weigh out the calculated amount of NHS-LC-biotin and add it to the antigen solution. Vortex gently to dissolve the NHS-LC-biotin.

Note: If the amount of NHS-LC-biotin required is too small to be weighed accurately, a stock solution of 2.5 mg/mL NHS-LC-biotin in Buffer A may be

prepared and an appropriate amount of this solution added to the antigen. This stock solution must be prepared fresh and used immediately.

6. Cover the reaction mixture tightly and mix gently on a tip plate or orbital mixer for 45 minutes at ambient temperature.

7. Add solid 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tis base) to a final concentration of 0.5M to the reaction mixture. Vortex gently to dissolve the Tris base. Incubate on a tip plate or orbital mixer for 10 minutes.

8. Apply the reaction mixture to the equilibrated desalting column (step 3) collecting suitably sized fractions.

9. Determine the OD280 of each collected fraction.

10. Pool the antigen containing fractions in a sterile screw capped container.

11. Determine the protein concentration of the biotinylated antigen by the Lowry method.

12. Store the biotinylated antigen at 4°C.

* * *

Although the methods and compositions of this invention have been illustrated by the foregoing examples and embodiments, the invention is not limited to only these examples and embodiments and modifications may be made without departing from the essence or essential characteristics of this invention. The scope of the invention is defined by the appended claims, and all compositions and methods that come within the meaning of the claims, either literally or by equivalence, are intended to be embraced therein.

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I CLAIM:

1. A composition comprising:

a. labeled molecules containing epitopes specific for an organism and non-specific proteins having epitopes that are found in the same organism; and

b. unlabeled molecules containing non-specific epitopes that compete with the non-specific epitopes on the labeled molecules for epitope binding sites on antibody molecules.

2. The composition of claim 1, wherein the epitopes specific for the organism are prepared by:

a. extracting proteins from a substantial portion of an organism;

b. fractionating the extracted proteins;

c. determining the concentration of each protein within each fraction; and

d. combining the proteins in quantities sufficient to produce an approximately equivalent end concentration of each protein in the composition.

3. The composition of claim 1, wherein the organism is selected from the group consisting of bacteria, chlamydia, mycoplasma, protozoa, rickettsia and viruses.

4. The composition of claim 1, wherein the organism is capable of infecting or colonizing a mammalian host.

5. The composition of claim 1, wherein the organism is a small multicellular organism.

6. The composition of claim 5, wherein the small multicellular

organism is an intracorporal parasite.

7. A method for preparing a reformulated protein mixture from a specific organism, said method comprising:

a. extracting proteins from a substantial portion of the entire organism;

b. fractionating the extracted proteins;

c. determining the concentration of each protein within each fraction; and

d. combining all the proteins in quantities sufficient to produce an approximately equivalent end concentration of each protein in the mixture.

8. The method of claim 7, further defined as:

e. labeling the reformulated protein mixture; and

f. adding to the mixture unlabeled non-specific proteins having epitopes homologous to the non-specific epitopes found on the labeled proteins.

9. A capture assay method to detect organism-specific immunoglobulins in a biological fluid sample, the capture assay method comprising:

a. attaching anti-immunoglobulin molecules to a support;

b. exposing the biological fluid sample to the support so that the immunoglobulins in the fluid can be captured by the anti-immunoglobulin molecules;

c. exposing the composition of claim 1 to the captured immunoglobulins under conditions suitable for formation of antigen

immunoglobulin complexes; and

d. measuring complexes of labeled molecules with the captured immunoglobulins, from which the presence in the biological fluid of immunoglobulins which are specific for the organism is inferred.

10. The capture assay method of claim 9, wherein the biological fluid sample is selected from the group consisting of whole blood, plasma, serum, sputum, urine, cerebrospinal fluid, intra-abdominal fluid, intrathoracic fluid, pericardial fluid, joint space fluid, pustular fluid, tear fluid, nasal secretions, sinus fluid, abscess fluid.

11. The capture assay method of claim 9, wherein the anti-immunoglobulin is anti-IgA.

12. The capture assay method of claim 9, wherein the anti-immunoglobulin is anti-IgD.

13. The capture assay method of claim 9, wherein the anti-immunoglobulin is anti-IgE.

14. The capture assay method of claim 9, wherein the anti-immunoglobulin is anti-IgG.

15. The capture assay method of claim 9, wherein the immunoglobulins in the fluid are IgE and IgG, and wherein the individually measured quantities of both are combined.

16. The capture assay method of claim 9, wherein the organism is *Helicobacter pylori.*

17. The capture assay of claim 9, wherein the organism is *Chlamydia pneumoniae.*

18. The capture assay method of claim 15, wherein the organism is *H. pylori.*

19. The capture assay method of claim 9, wherein serum IgE and IgG are measured in a biological sample obtained both before and after treatment of a subject to eradicate the organism.

20. The capture assay method of claim 19. further defined as combining the measured IgE and IgG values and comparing them to determine whether the organism is present.

21. The capture assay method of claim 19, wherein the organism is *H. pylori.*

22. The capture assay method of claim 19, wherein the organism is *Chlamydia pneumoniae.*

23. The capture assay method of claim 15, wherein the IgE and IgG quantities are combined by multiplying the quantity of specific IgE by the quantity of IgG corresponding to each biological fluid sample tested; and comparing the multiplied IgE x IgG values with a standard value to determine whether the organism is present.

24. The capture assay method of claim 23, wherein the organism is *H. pylori.*

25. A capture assay method to determine effects of treatment to eradicate an organism in a subject, said method comprising from a biological fluid sample obtained from the subject:

a. obtaining a value for a quantity of organism-specific IgG before treatment;

b. obtaining a value for the quantity of organism-specific IgG after

treatment;

c. obtaining a value of organism-specific IgE before treatment;

d. obtaining a value of organism-specific IgE after treatment;

e. combining the values obtained in step a with those obtained in step c;

f. combining the values obtained in step b with those obtained in step d; and

g. inferring whether treatment is successful by determining whether the combined value in step f is less than in step e.

26. The capture assay method of claim 25, wherein the combined values are IgG multiplied by IgE.

27. The capture assay method of claim 26, wherein the value in step f is not more than 40% of the value of step e from which treatment is inferred to be successful.

28. A kit comprising a support and, in separate containers, calibrator solutions and a labeled reformulated antigen mixture containing quenching antigens.

29. The kit of claim 28, further defined as having a means to detect a complex between the labeled reformulated antigen mixture and an antibody in a biological fluid sample.

30. A composition comprising:

a. labeled molecules containing epitopes specific for an allergen and non-specific epitopes that are found in the same allergen; and

b. unlabeled molecules containing non-specific epitopes that compete with the non-specific epitopes on the labeled molecules for epitope binding sites on antibody molecules.

31. The composition of claim 30, wherein the epitopes specific for the allergen are prepared by:

a. extracting proteins from a substantial portion of an allergen;

b. fractionating the extracted proteins;

c. determining the concentration of each protein within each fraction; and

d. combining the proteins in quantities sufficient to produce an approximately equivalent end concentration of each protein in the composition; and

e. using the composition to provide specific epitopes.

32. The composition of claim 30, wherein the allergen is selected form the group consisting of bacteria, chlamydia, mycoplasma, protozoa, rickettsia, viruses, pollens, epidermal agents, mold spores, foods, venoms and allergenic pharmaceutical agents.

33. The composition of claim 32, wherein the pollens comprise Orchard Grass Pollen, Brome Grass Pollen, Giant Ragweed Pollen, Pigweed Pollen, Smooth Alder Pollen and River Birch Pollen.

34. The composition of claim 30, wherein the allergen is a small multicellular organism.

35. The composition of claim 34, wherein the small multicellular organism is an intracorporal parasite.

36. A method for preparing a reformulated protein mixture from a specific allergen, said method comprising:

a. extracting proteins from a substantial portion of the entire allergen;

b. fractionating the extracted proteins;

c. determining the concentration of each protein within each fraction; and

d. combining all the proteins in quantities sufficient to produce a mixture comprising an approximately equivalent end concentration of each protein.

37. The method of claim 36, further defined as:

e. labeling the reformulated protein mixture; and

f. adding to the mixture an unlabeled mixture of non-specific proteins having epitopes homologous to non-specific epitopes found on the labeled proteins.

38. A capture assay method to detect allergen-specific immunoglobulin molecules in a biological fluid sample, the capture assay method comprising:

a. attaching anti-immunoglobulin molecules to a support;

b. exposing the biological fluid sample to the support so that the immunoglobulin molecules in the fluid can be captured by the anti-immunoglobulin molecules;

c. exposing the composition of claim 30 to the captured immunoglobulin molecules under conditions suitable for formation of antigen immunoglobulin complexes; and

d. measuring the complexes of labeled molecules with the captured immunoglobulins, from which the presence in the biological fluid of immunoglobulins which are specific for the allergen is inferred.

39. The capture assay method of claim 38, wherein the biological fluid sample is selected from the group consisting of whole blood, plasma, serum, sputum, urine, cerebrospinal fluid, intra-abdominal fluid, intrathoracic fluid, pericardial fluid, joint space fluid, pustular fluid, tear fluid, nasal secretions, sinus fluid, and abscess fluid.

40. The capture assay method of claim 38, wherein the anti-immunoglobulin is anti-IgA.

41. The capture assay method of claim 38, wherein the anti-immunoglobulin is anti-IgD.

42. The capture assay method of claim 38, wherein the anti-immunoglobulin is anti-IgA.

43. The capture assay method of claim 38, wherein the anti-immunoglobulin is anti-IgG.

44. The capture assay method of claim 38, wherein the immunoglobulins in the biological fluid sample are IgE and IgG, and wherein the individually measured quantities of both are combined.

45. A capture assay method to detect allergen-specific immunoglobulin molecules in a biological fluid sample, the capture assay method comprising:

a. covalently attaching anti-immunoglobulin molecules to a support;

b. exposing the biological fluid sample to the support for a period of time so that the immunoglobulin molecules in the fluid can be captured by the anti-immunoglobulin molecules;

c. exposing a biotinylated antigen composition to the captured immunoglobulin molecules minutes so that suitable antigen-immunoglobulin complexes are formed;

d. contacting the antigen-immunoglobulin complexes with a labeled avidin-like molecule so that the labeled molecule can specifically attach to any biotin on the antigen-immunoglobulin complex; and

e. measuring the quantity of label within the captured antigen-immunoglobulin complex, from which the presence in the biological fluid of immunoglobulin molecules which are specific for a disease causing organism or molecule is inferred.

46. The capture assay method of claim 45, wherein the biological fluid is exposed to the support for a period of time between 5 minutes and 24 hours.

47. The capture assay method of claim 45, wherein the biotinylated antigen composition is exposed to the captured immunoglobulin molecules for a period of time between 5 minutes and 180 minutes.

48. The capture assay method of claim 45 wherein the complexes are contacted with a labeled avidin-like molecule for a period of time between 2 and 180 minutes.

49. The capture assay method of claim 43, wherein the biological fluid sample is selected from the group consisting of whole blood, plasma, serum, sputum, urine, cerebrospinal fluid, intra-abdominal fluid, intrathoracic fluid, pericardial fluid, joint space fluid, pustular fluid, tear fluid, nasal secretions, sinus fluid, and abscess fluid.

50. The capture assay method of claim 45, wherein the anti-immunoglobulin is anti-IgA.

51. The capture assay method of claim 45, wherein the anti-immunoglobulin is anti-IgD.

52. The capture assay method of claim 45, wherein the anti-immunoglobulin is anti-IgE.

53. The capture assay method of claim 45, wherein the anti-immunoglobulin is anti-IgG.

54. The capture assay method of claim 45 wherein the biotinylated antigen is derived from a bacteria, chlamydia, mycoplasma, protozoa, rickettsia, virus, pollen, epidermal agent, mold spore, food, venom and allergenic pharmaceutical agent.

55. A capture assay method to detect allergen-specific immunoglobulins in a biological fluid sample, the capture assay method comprising:

a. attaching strongly anti-immunoglobulin molecules to a support;

b. exposing the biological fluid sample to the support so for a period of time between 5 minutes and 24 hours so that the immunoglobulins in the fluid can be captured by the anti-immunoglobulin molecules;

c. exposing a biotinylated antigen composition to the captured immunoglobulin molecules for a period of time between 5 minutes and 180 minutes so that suitable antigen-immunoglobulin complexes are formed;

d. contacting the antigen-immunoglobulin complexes with a labeled avidin-like molecule for a period of time between 2 and 180 minutes so that the labeled molecule can specifically attach to any biotin present on the antigen-immunoglobulin complex; and

e. measuring the quantity of label within the captured antigen-

immunoglobulin complex, from which the presence in the biological fluid of immunoglobulin molecules which are specific for a disease-causing organism or molecule is inferred.

56. The capture assay method of claim 55, wherein the biological fluid sample is selected from the group consisting of whole blood, plasma, serum, sputum, urine, cerebrospinal fluid, intra-abdominal fluid, intrathoracic fluid, pericardial fluid, joint space fluid, pustular fluid, tear fluid, nasal secretions, sinus fluid, and abscess fluid.

57. The capture assay method of claim 55, wherein the anti-immunoglobulin is anti-IgA.

58. The capture assay method of claim 55, wherein the anti-immunoglobulin is anti-IgD.

59. The capture assay method of claim 55, wherein the anti-immunoglobulin is anti-IgE.

60. The capture assay method of claim 55, wherein the anti-immunoglobulin is anti-IgG.

61. The capture assay method of claim 55, wherein the biotinylated antigen is derived from a bacteria, chlamydia, mycoplasma, protozoa, rickettsia, virus, pollen, epidermal agent, mold spore, food, venom and allergenic pharmaceutical agent.

62. A capture assay method to detect allergen-specific immunoglobulins in a biological fluid sample, the capture assay method comprising:

a. covalently attaching anti-immunoglobulin molecules to a support;

b. exposing the biological fluid sample to the support for a period of

time between about 5 minutes and 24 hours so that the immunoglobulins in the fluid can be captured by the anti-immunoglobulin molecules;

c. exposing a biotinylated antigen composition to the captured immunoglobulins for a period of time between about 5 minutes and 180 minutes so that suitable antigen-immunoglobulin complexes are formed;

d. contacting the antigen-immunoglobulin complexes with an avidin-like molecule conjugated to a chemiluminogenic, chromogenic or fluorogenic enzyme for a period of time between about 2 and 180 minutes so that the labeled molecular complex can specifically attach to any biotin present on the antigen-immunoglobulin complex;

e. contacting the bound molecular complexes, for a time between 2 and 240 minutes, with a solution of substrate which undergoes reaction in the presence of the enzyme to yield a colored, chemiluminescent or fluorescent product;

f. measuring the color, chemiluminescence or fluorescence product; and

g. determining the amount of organism-specific or antigen-specific immunoglobulin in the biological fluid by comparing the color, chemiluminescence or fluorescence level determined in step (f) with those of control solutions.

63. The capture assay method of claim 62, wherein the biological fluid sample is selected from the group consisting of whole blood, plasma, serum, sputum, urine, cerebrospinal fluid, intra-abdominal fluid, intrathoracic fluid, pericardial fluid, joint space fluid, pustular fluid, tear fluid, nasal secretions, sinus fluid, and abscess fluid.

64. The capture assay method of claim 62, wherein the anti-

immunoglobulin is anti-IgA.

65. The capture assay method of claim 62, wherein the anti-immunoglobulin is anti-IgD.

66. The capture assay method of claim 62, wherein the anti-immunoglobulin is anti-IgE.

67. The capture assay method of claim 62, wherein the anti-immunoglobulin is anti-IgG.

68. The capture assay method of claim 62, wherein the biotinylated antigen is derived from a bacteria, chlamydia, mycoplasma, protozoa, rickettsia, virus, pollen, epidermal agent, mold spore, food, venom and allergenic pharmaceutical agent.

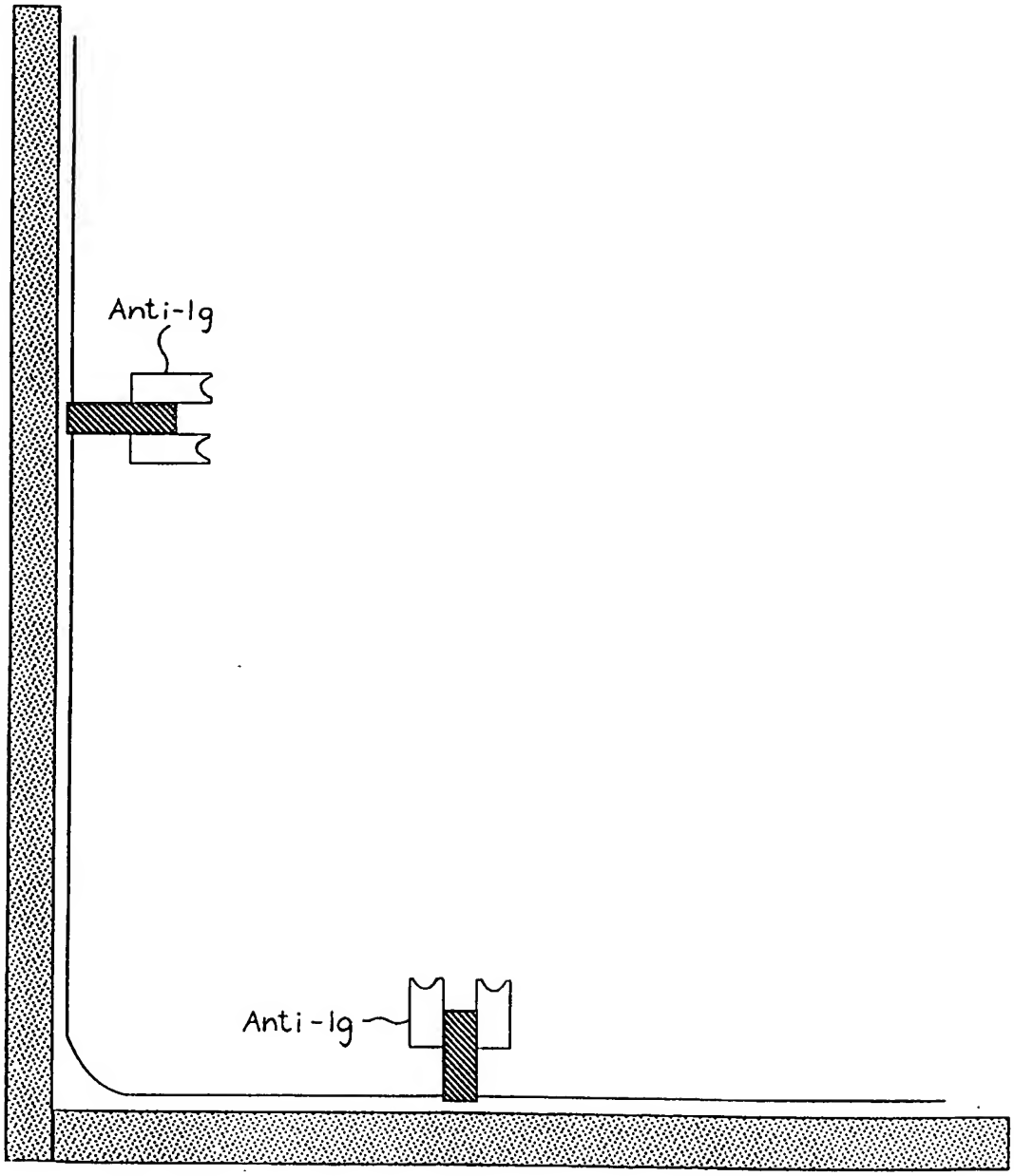


Figure 1

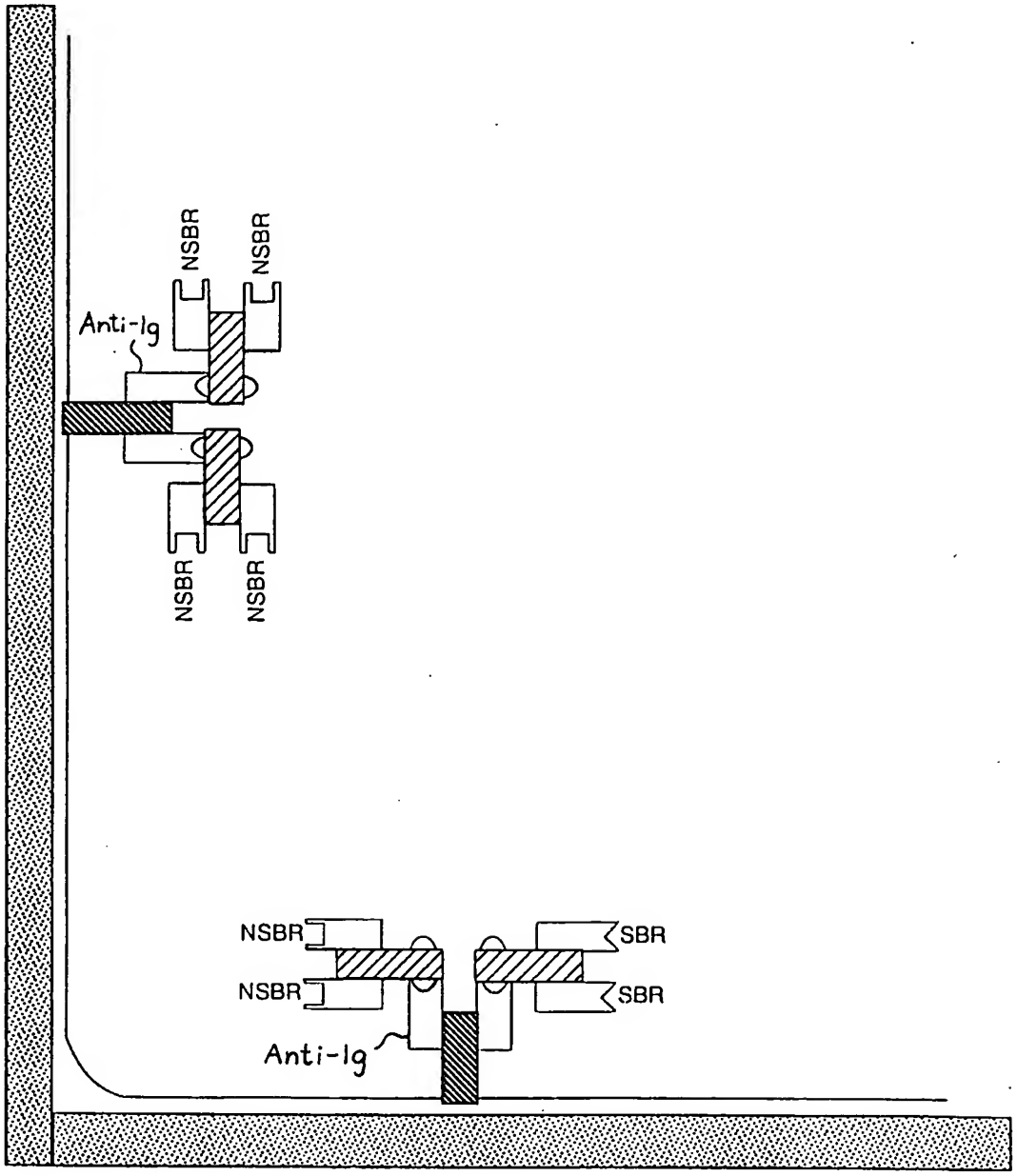


Figure 2

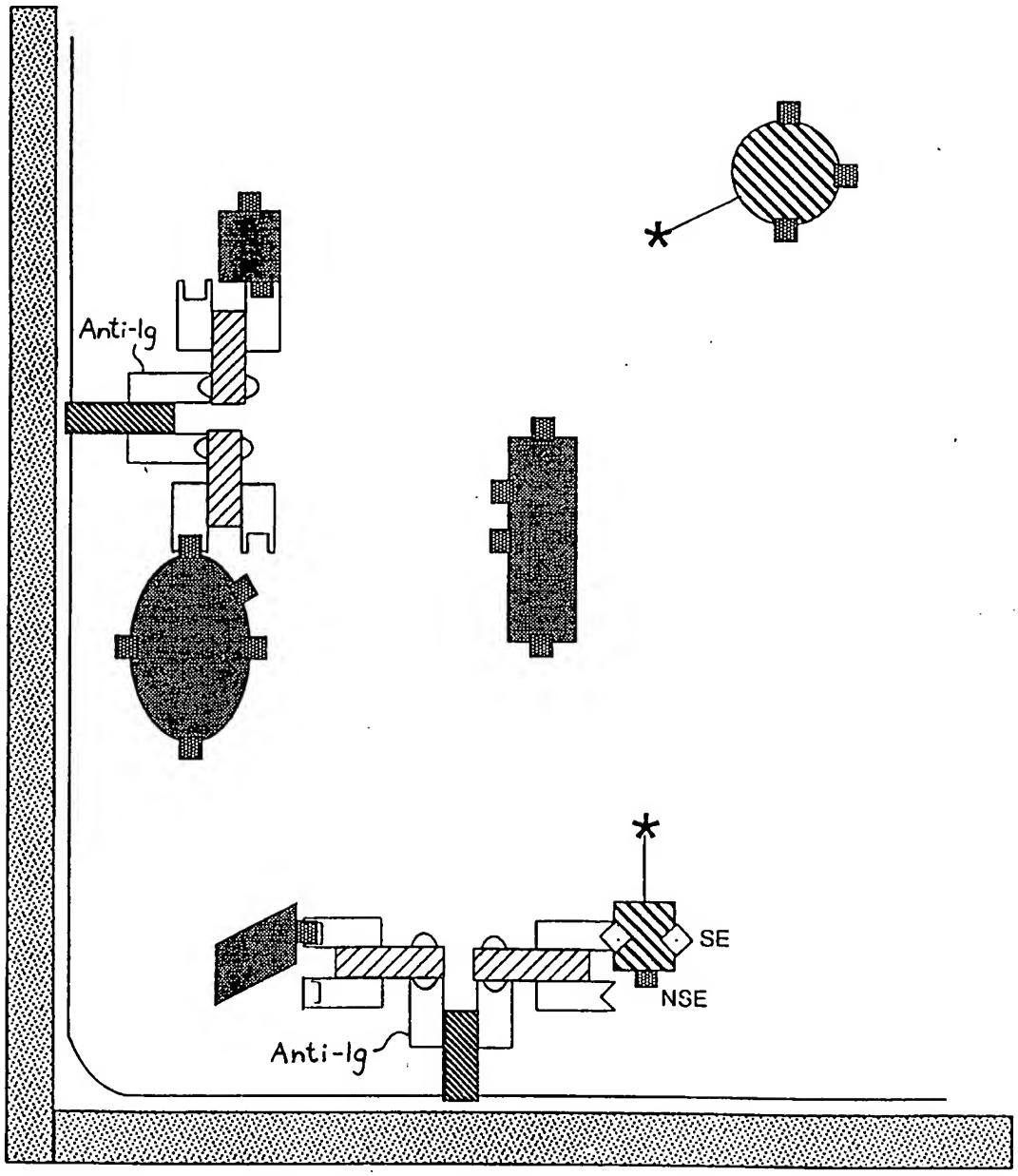


Figure 3

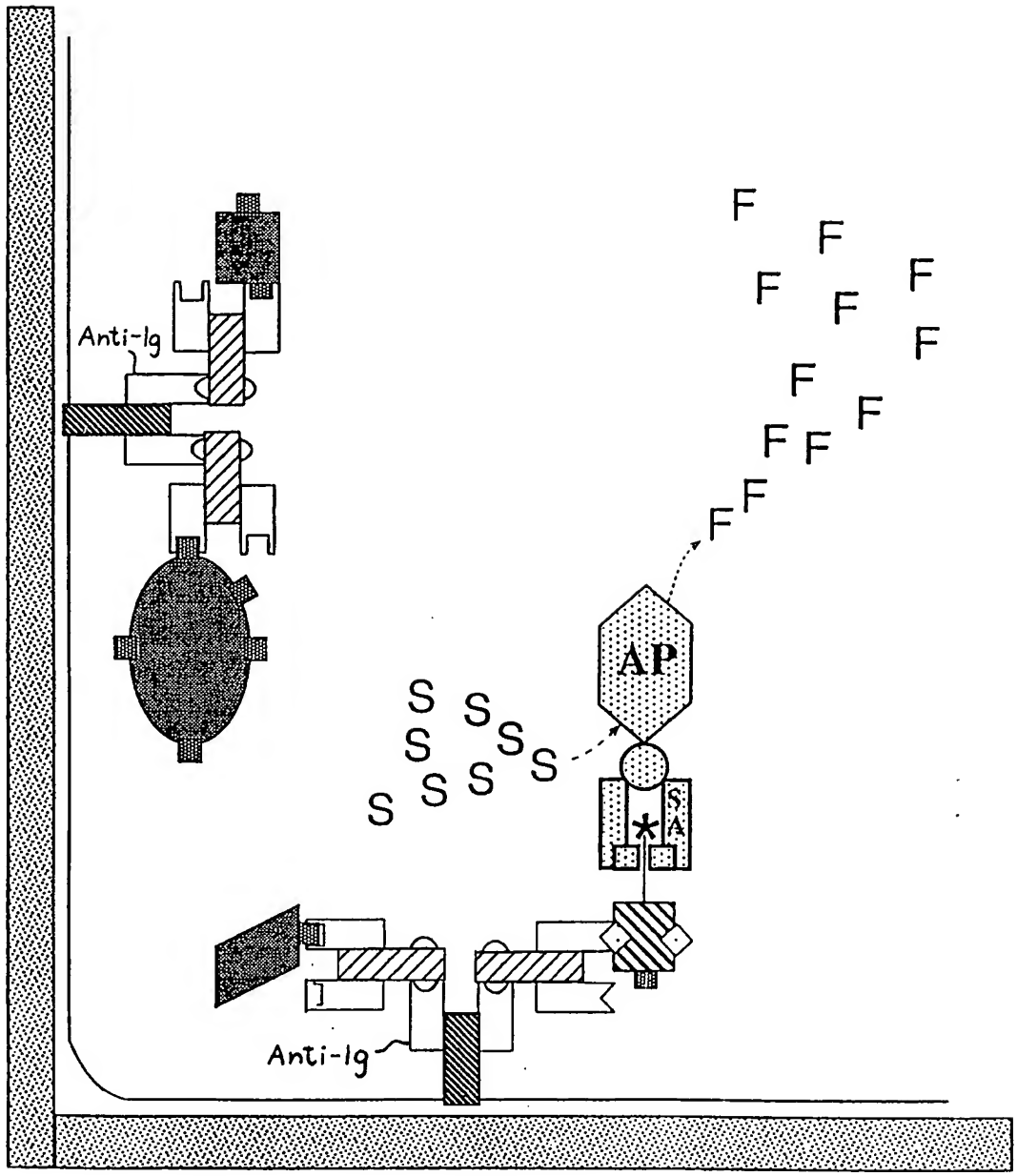


Figure 4

Figure 5

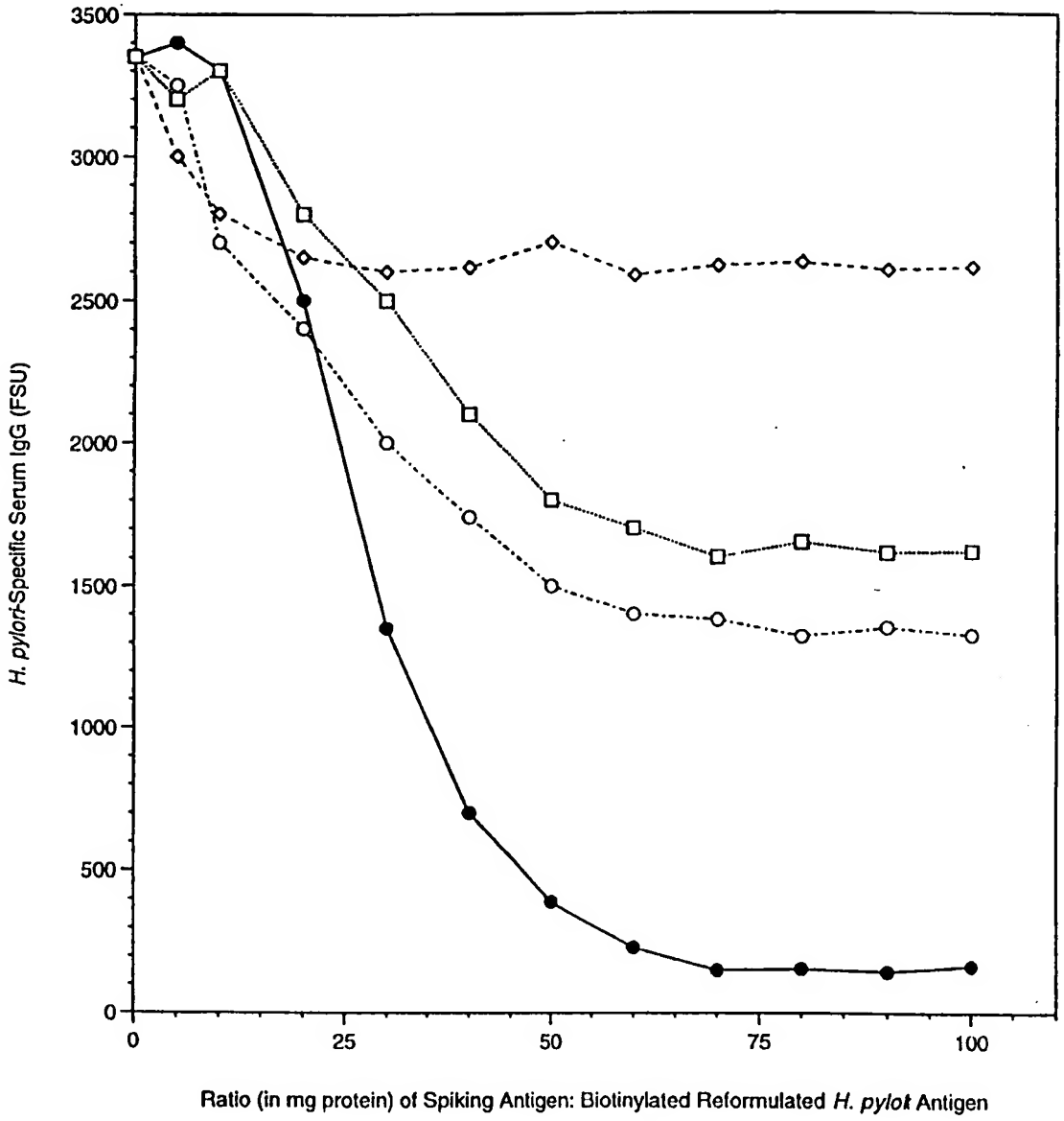

3500
3000
2500
2000
1500
1000
500
0
0
25
50
75
100
H. pylori-Specific Serum IgG (FSU)
Ratio (in mg protein) of Spiking Antigen: Biotinylated Reformulated H. pylori Antigen

Figure 6

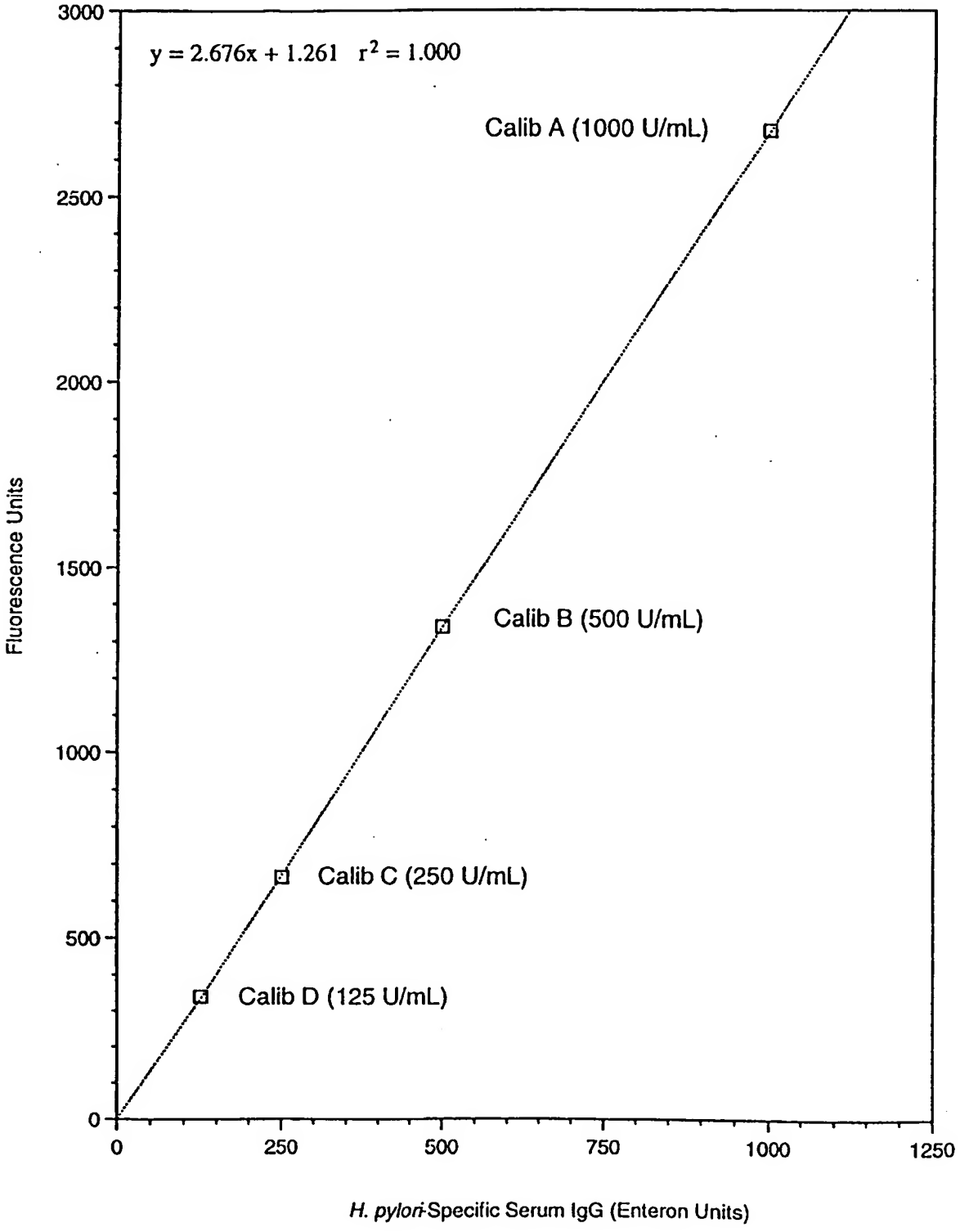

y = 2.676x + 1.261 r² = 1.000
Calib A (1000 U/mL)
Calib B (500 U/mL)
Calib C (250 U/mL)
Calib D (125 U/mL)
Fluorescence Units
3000
2500
2000
1500
1000
500
0
0
250
500
750
1000
1250
H. pylori-Specific Serum IgG (Enteron Units)

Figure 7

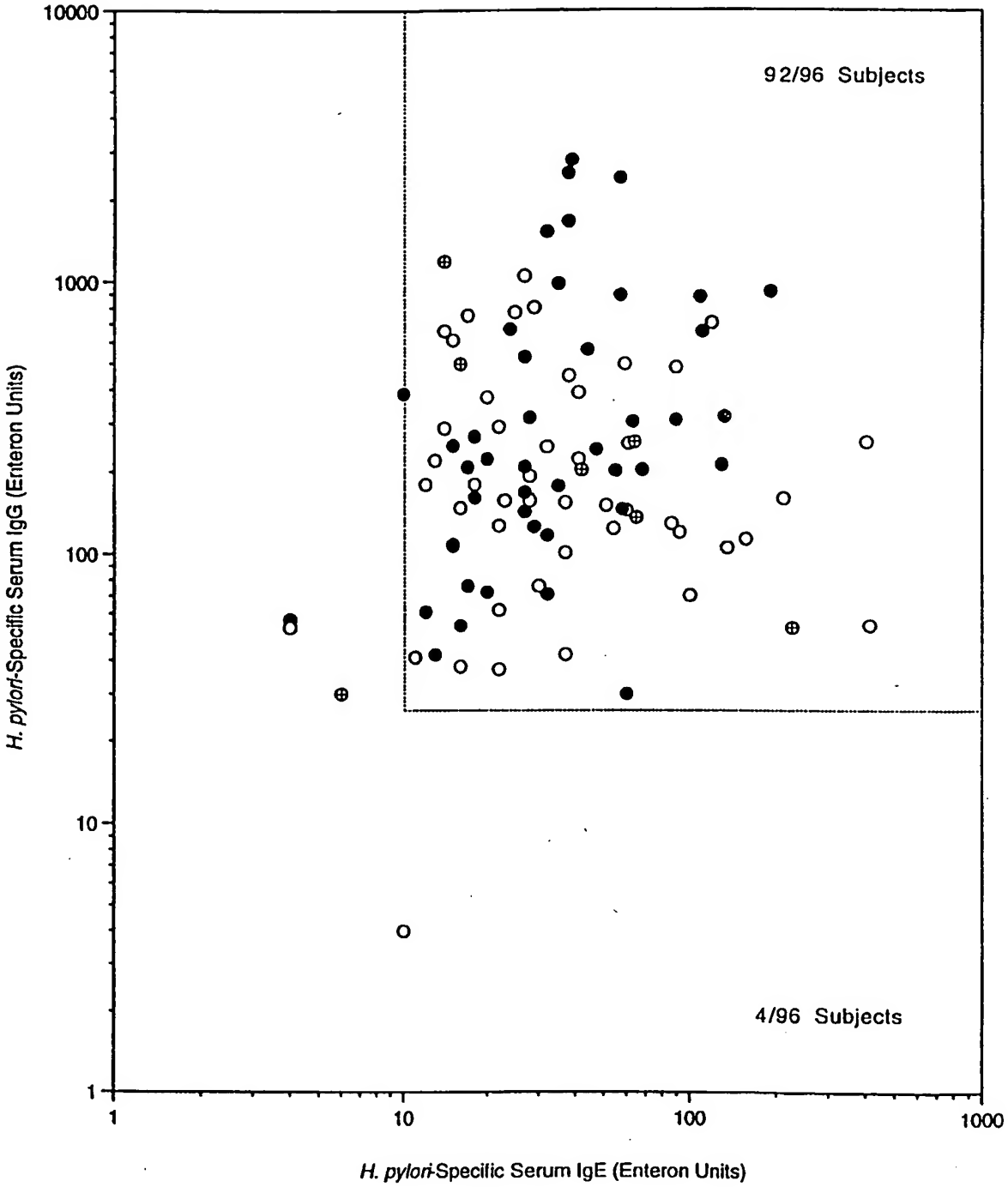

10000
1000
100
10
1
1
10
100
1000
92/96 Subjects
4/96 Subjects
H. pylori-Specific Serum IgG (Enteron Units)
H. pylori-Specific Serum IgE (Enteron Units)

Figure 8

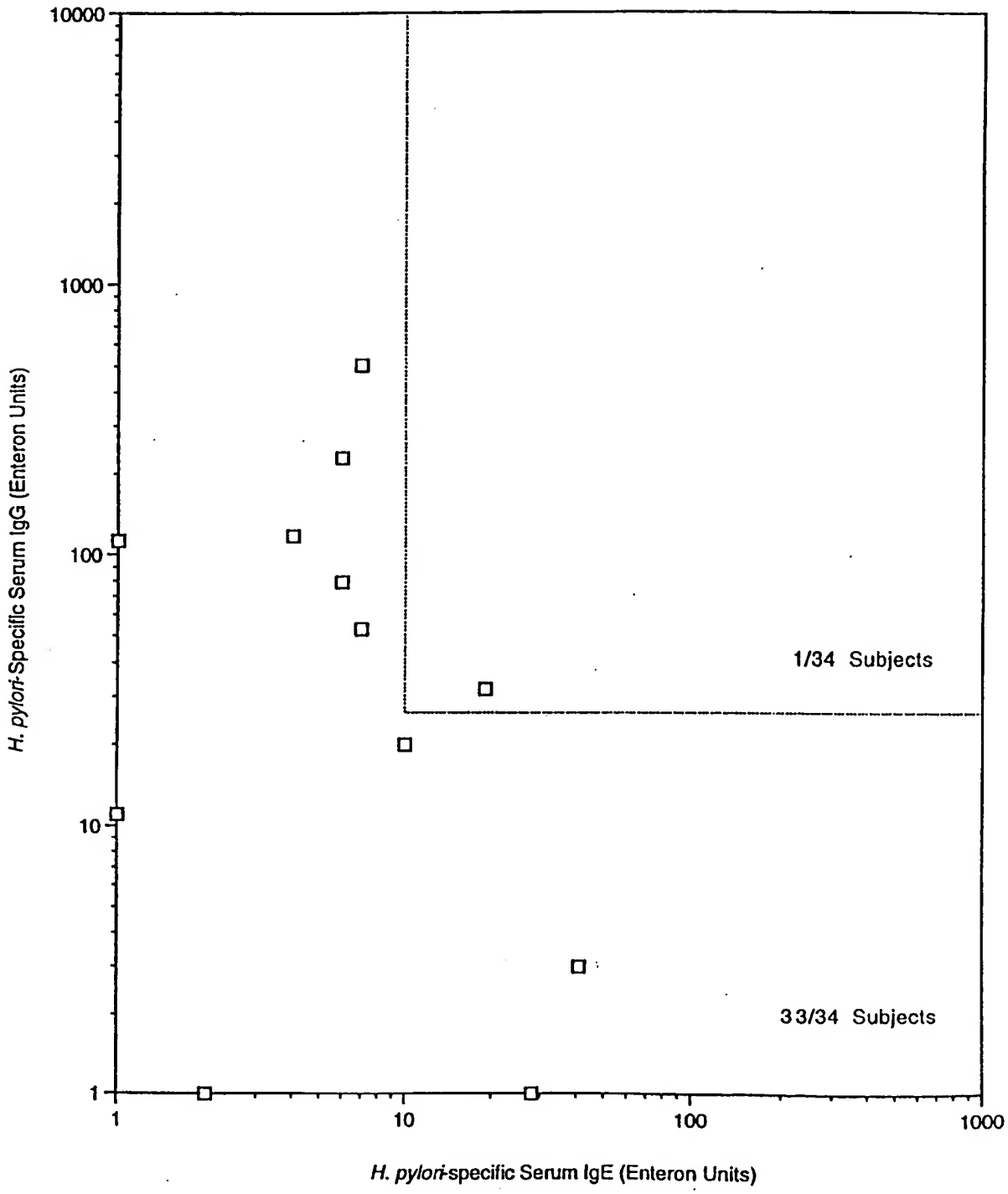

10000
1000
100
10
1
1
10
100
1000
H. pylori-Specific Serum IgG (Enteron Units)
H. pylori-specific Serum IgE (Enteron Units)
1/34 Subjects
33/34 Subjects

Figure 9

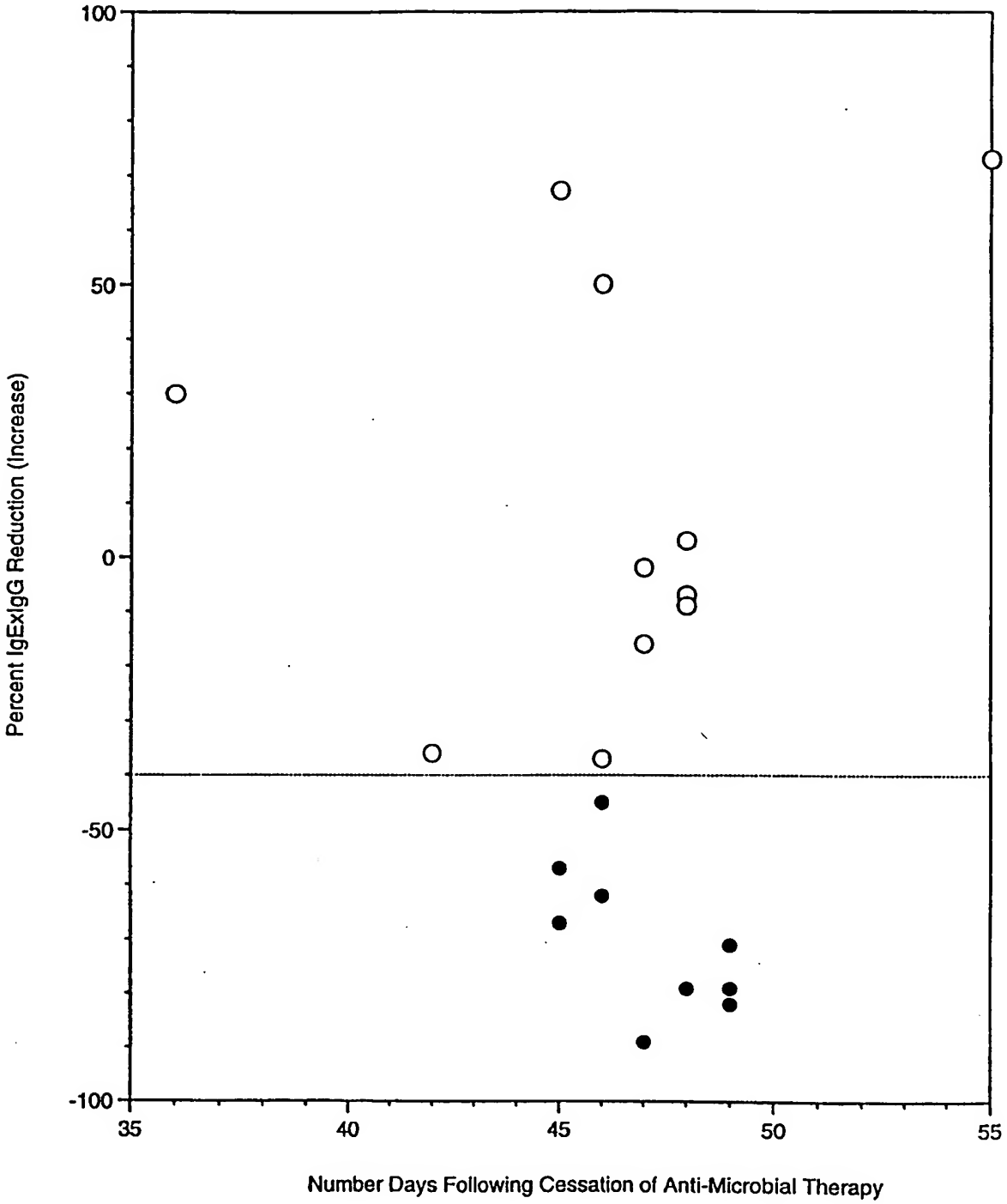

100
50
0
-50
-100
35
40
45
50
55
Percent IgExIgG Reduction (Increase)
Number Days Following Cessation of Anti-Microbial Therapy

Figure 10

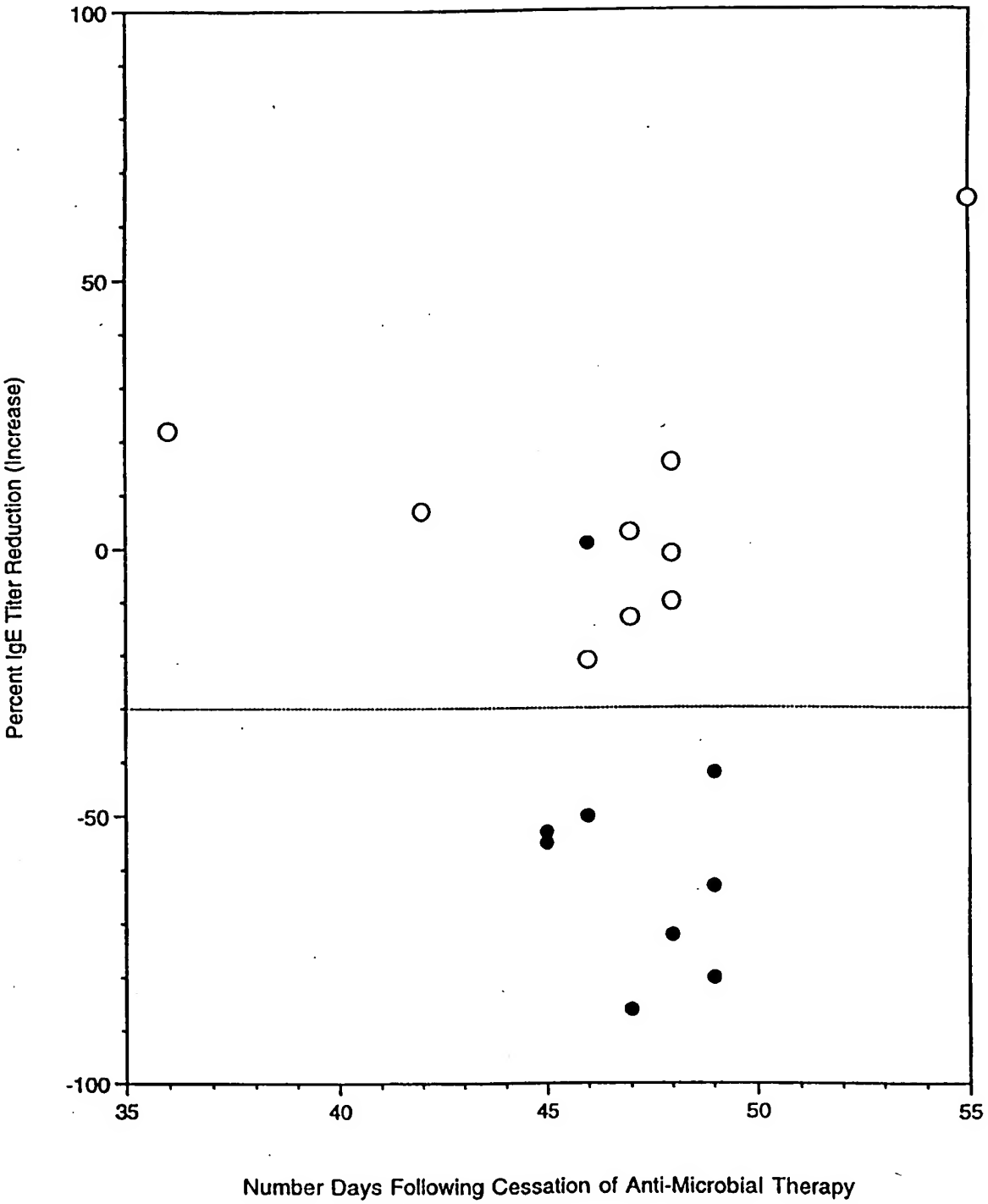
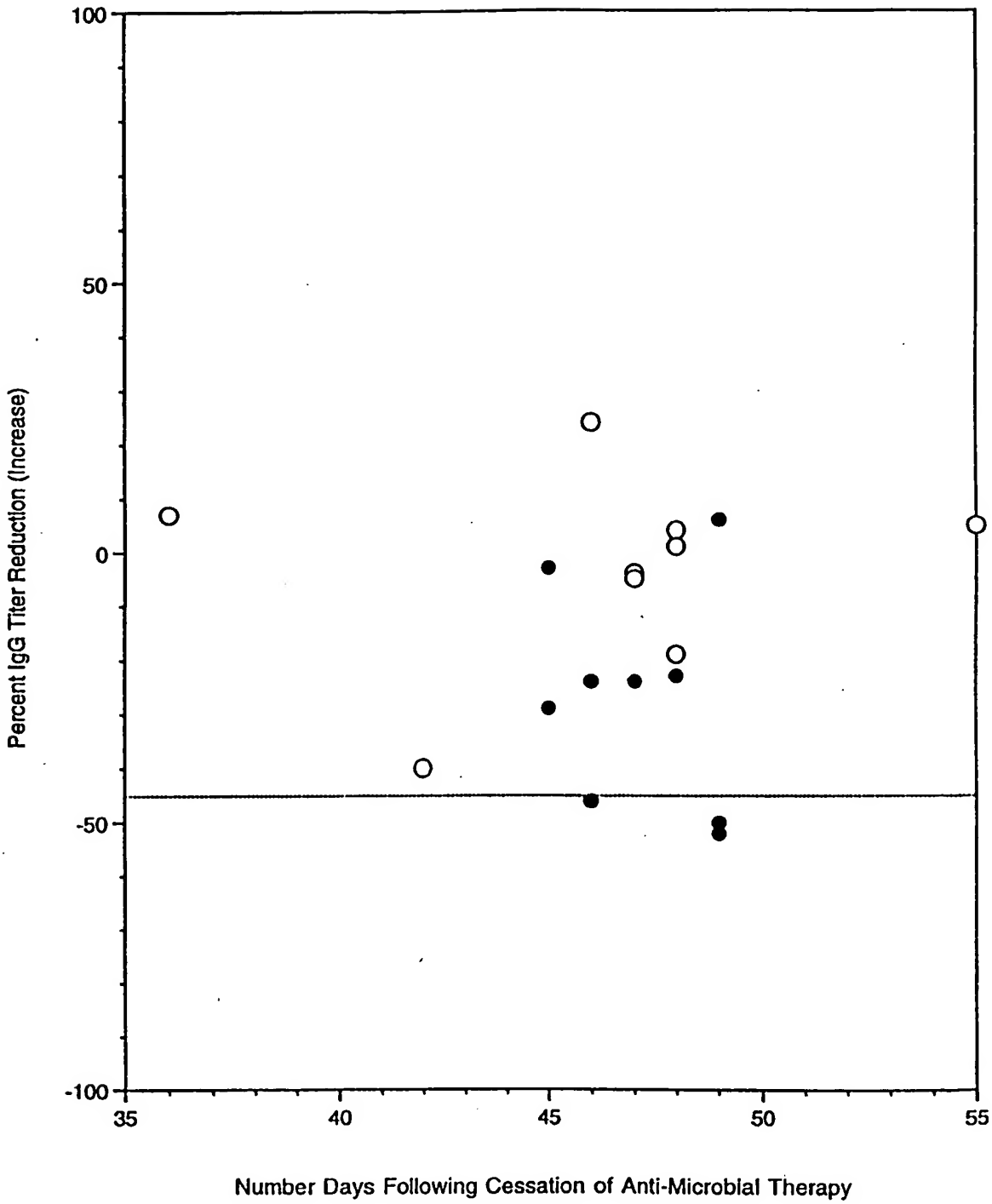

100
50
0
-50
-100
35
40
45
50
55
Percent IgE Titer Reduction (Increase)
Number Days Following Cessation of Anti-Microbial Therapy

Figure 11


100
50
0
-50
-100
Percent IgG Titer Reduction (Increase)
35
40
45
50
55
Number Days Following Cessation of Anti-Microbial Therapy

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/18588

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/53 G01N33/543 G01N33/569 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 278 340 A (NAOT YEHUDITH ;DIMOTECH LTD (IL)) 17 August 1988 see the whole document ---	1-68
A	WO 96 26740 A (ENTERON LIMITED PARTNERSHIP) 6 September 1996 see the whole document ---	1-68
A	FR 2 556 840 A (IMMUNOTECH SA) 21 June 1985 see the whole document ---	1-68

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 February 1998	13/02/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Hoekstra, S

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/18588

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CALENOFF E ET AL: "Bacteria-specific IgE in patients with nasal polyposis. A preliminary report." ARCHIVES OF OTOLARYNGOLOGY, vol. 109, no. 6, 1983, pages 372-375, XP000575015 see the whole document ---	1-68
A	CALENOFF E ET AL: "SERUM IMMUNOGLOBULIN E DIRECTED AGAINST HELICOBACTER PYLORI PROTEINS WITH GASTRIC AND DUODENAL ULCERS" GASTROENTEROLOGY, vol. 108, no. 4, 1 April 1995, page A64 XP000575009 -----	1-68

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte ional Application No
PCT/US 97/18588

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		DE 3882727 T	02-12-93
		JP 1257264 A	13-10-89
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FR 2556840 A	21-06-85	NONE	